CHARACTERIZATION OF THE INTERACTION OF THE TERMINAL COMPLEMENT COMPLEXES WITH A MODEL MEMBRANE SYSTEM: COMPARISON OF THE CHANNEL PROPERTIES OF C5-8 AND C5-9 LESIONS

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Title of Thesis: Characterization of the Interaction of the Terminal Complement Complexes with a Model Membrane System: Comparison of the Channel Properties of C5-8 and C5-9 Lesions

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Doctor of Philosophy Degree
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ABSTRACT

Title of Thesis: Characterization of the Interaction of the Terminal Complement Complexes with a Model Membrane System: Comparison of the Channel Properties of C5-8 and C5-9 Lesions

Rikki Solow, Doctor of Philosophy, 1987

Thesis directed by: Valerie W. Hu, Ph.D.
Department of Biochemistry

The interaction of C5-8 and C5-9 complexes with model membranes was investigated using large unilamellar vesicles (LUV's) prepared by reverse phase evaporation. In particular, C5-8 and C5-9 channel activity with regard to channel pore size, mode of marker release and dynamics of channel formation, was characterized. This was achieved by measuring the release of entrapped fluorescent dye 6-carboxy-fluorescein (6CF), and/or radiolabeled markers, from complement-treated LUV's.

C5-8- and C5-9-induced permeability changes and the respective pore sizes of the complexes in LUV membranes were similar to those induced by complement in natural membranes, suggesting that the lesions formed on these model membranes by C5-8 and C5-9

complexes are probably identical to those formed on biological membranes. In comparing the mode of marker release from LUV's, it was of particular interest that C5-9 released dye from vesicles in an all-or-none manner whereas C5-8 complexes induced only a graded or partial dye release. This result suggested that, while C5-9 complexes formed stable channels in membranes, the C5-8 complexes formed transient or unstable channels. It was further shown that the conversion of the transient C5-8 channel to a stable C5-9 channel and the enlargement of the pore size of the C5-8 complex by C9 molecules were temperature-dependent processes. These data suggest that the mechanism of C5-8- and C5-9-induced changes in membrane permeability are qualitatively different.

This difference is also reflected in the kinetics of marker release by the C5-8 and C5-9 channels. Comparison of the rate of dye release with the rate of C8 insertion into the membrane bilayer revealed a lag time between the completion of C8 insertion and the endpoint of 6CF release from vesicles, indicative of a post-insertional process required for formation of the C5-8 channel. A post-insertional step in the formation of the C5-9 channel is also suggested by the fact that C9 (and C8) insertion occurred at low temperatures even though no

dye was released. These latter data indicate that, though the C5-8 and C5-9 complexes exhibit quite different channel properties, the functional activity of both types of channels is dependent on one or more temperature-sensitive, post-insertional process(es).

CHARACTERIZATION OF THE INTERACTION OF THE TERMINAL COMPLEMENT COMPLEXES WITH A MODEL MEMBRANE SYSTEM: COMPARISON OF THE CHANNEL PROPERTIES OF C5-8 AND C5-9 LESIONS

by

Rikki Solow

Thesis submitted to the Faculty of the Department of Biochemistry Graduate Program of the Uniformed Services Univeristy of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1987

DEDICATION

To Valerie --- for providing an excellent, encouraging, yet stimulating and challenging environment in which to develop proper scientific technique; it has been my good fortune and privilege to have, as my advisor, both a teacher and a friend.

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Abbreviations: LUV's, large unilamellar vesicles; 6CF, 6-carboxyfluorescein; 12-APS-GlcN, 12-(4-azido-2-nitrophenoxy)stearoyl[1-^{14}C]glucosamine; RBC's, red blood cells; MLV's, multilamellar vesicles; SUV's, small unilamellar vesicles; DML, dimyristoyllecithin; EYL, egg yolk lecithin; DPL, dipalmitoylphosphatidyl-choline; PBS, phosphate buffered saline; diS-C_3-(5), 3,3' - dipropylthiodicarbocyanine iodide; Brij, polyoxyethylene 20 cetyl ether; ^{3}H-NSP, N-succinimidyl-[2, 3-^{3}H]proprionate; EM, electron microscopy.

INTRODUCTION

The complement system, which consists of at least 20 serum proteins, constitutes a major part of the humoral immune system (Figure 1A) (1). Activation of the complement cascade results in:

(1) the generation of complement cleavage products with varied biological activity, such as increasing vascular permeability, inducing histamine release from mast cells, smooth muscle contraction, and monocyte recruitment.

(2) the opsonization of bacteria by complement fission products which facilitate phagocytosis.

(3) the formation of a membrane attack complex by the late-acting complement components which leads to the death or disruption of a variety of targets, such as bacteria, viruses, virus-infected cells, fungi, parasites and some tumor cells.

One of the main functions of complement activation involves the transfer of the late or terminal complement components, C5-9, from the aqueous

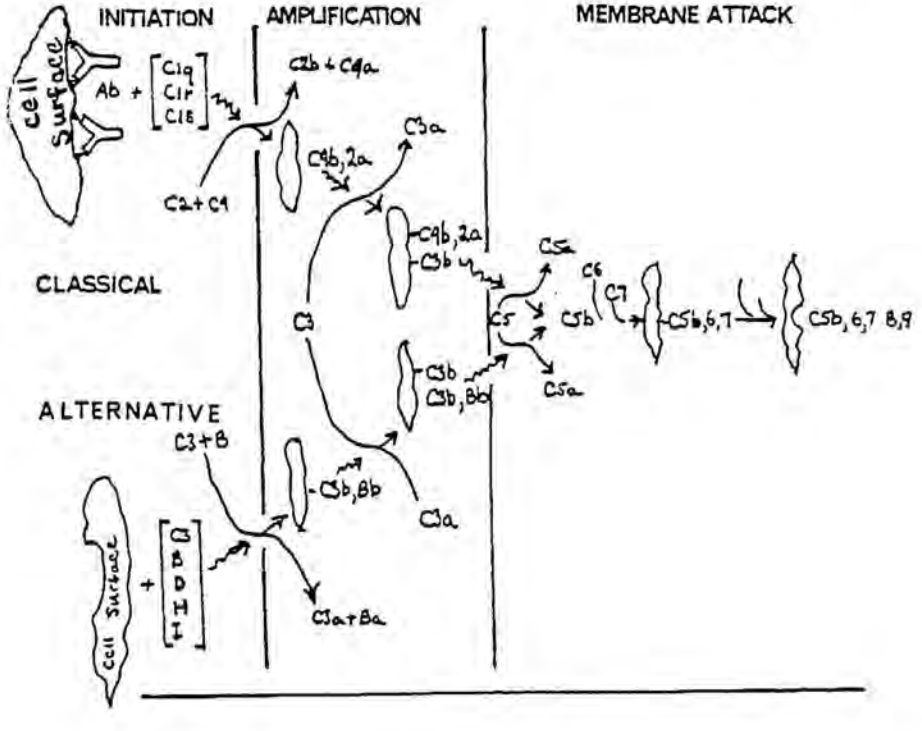
Figure 1

The Complement System: (A) Proteins of the complement system. (B) Schematic representation of the two pathways of complement activation leading to the formation of the membrane attack complex, C5-9. Figure 1 was adapted from: Immunology, edited by Joseph A. Bellanti, W.B. Saunders Company (1985).

A

PROTEIN	MOLECULAR WEIGHT
Classical Pathway	
C1q	400,000
C1r	190,000
C1s	88,000
C4	200,000
C2	117,000
C3	185,000
Alternative Pathway	
Factor B	93,000
Factor D	23,000
C3	185,000
Membrane Attack Pathway	
C5	200,000
C6	128,000
C7	121,000
C8	154,000
C9	79,000
Regulatory Proteins	
C1 Inhibitor	85,000
Carboxypeptidase N	310,000
Factor H	150,000
Factor I	80,000
Properdin	180,000
S-Protein	71,000

B



phase (serum) to the surface of the target cell, resulting in the assembly of a transmembrane lesion which ultimately causes lysis of the cell via colloid osmotic shock.

As shown in Figure 1B, activation of the complement cascade can occur by two pathways, called "the classical" and "the alternative" (2). The initiation event of the classical pathway involves the activation of the C1 protein complex upon binding to Ag-Ab complexes on cell surfaces. This is followed by a series of enzymatic cleavages which result ultimately in the assembly of a trimeric proteolytic complex consisting of C4b, C2a, and C3b. The function of this complex, called the C5 convertase, is to cleave C5 into C5a and C5b. C5b can then bind C6 and initiate the assembly of the C5-9 membrane attack complex.

Activation of complement via the alternative pathway involves direct activation and binding of C3b on a suitable surface thereby bypassing the early-acting components C_1, C_4 and C_2 (3). In the presence of properdin and Mg^{++}, surface-bound C3b can bind factor Bb to generate a C5 convertase consisting of C3bBbC3b and initiate the formation of the membrane attack phase.

Despite different modes of activation, both the classical and alternative pathway can result in the

self-assembly of the membrane attack complex involving the terminal components, C5-9, on the target membranes. In the self-assembly process, the cleavage of C5 to C5b by C5 convertase (4) is followed by the binding of C5b to C6 in the fluid phase (5). The C5b-6 complex then forms a reversible, low affinity association with the membrane (6). Upon binding of C7 to C5b-6, a conformational change occurs, which exposes hydrophobic binding sites on the proteins and allows a stable, irreversible, membrane-bound C5-7 complex to form (7). Subsequent binding of C8 and C9 to the C5-7 complex generates the C5-9 membrane lesion. The formation of these C5-9 lesions have been shown in studies using red blood cells (RBC's) to be correlated with the rapid exchange of ions, swelling of the cell and lysis or cell death (colloid osmotic shock) (8).

Using model membranes composed of phospholipid and cholesterol, Haxby et al.(9) demonstrated that the lipid bilayer was the substrate of complement attack. Furthermore, complement could induce ^{14}C-glucose release from liposomes made with phospholipid analogs which were not susceptible to phospholipases (10). These results showed that the mechanism of complement-mediated lysis did not involve cellular proteins.

Thompson and Lachman (11) showed that the lytic agent in complement lysis was the C5-9 complex which

could be assembled on membranes in the absence of the earlier complement components. Similar results have been obtained with bacteria and liposomes (12, 13). This form of lysis, mediated only by the terminal proteins without activation of earlier complement proteins, was called "reactive lysis."

Similarly, formation of membrane-bound C5-8 complexes assembled in the absence of C9, have been shown to cause lysis of RBC's and to induce permeability changes in RBC ghosts (14-16). However, C5-8 lytic activity differed qualitatively and quantitatively from that induced by C5-9. Treatment of RBC and RBC ghosts by C5-8 resulted in a significantly slower rate of lysis and marker release than that which resulted from treatment with C5-9. The time required for lysis to cease from C5-8-treated RBC's was 150 minutes compared to the 90 minutes required for that of C5-9-treated RBC's (14). In addition, more C5-8 than C5-9 complexes were required to lyse these cells (17), which suggested that more than one C5-8 complex per cell was necessary in order to induce lysis. Interestingly, humans lacking C9 in their serum have apparently normal humoral immunity, while those deficient in C8 are prone to frequent, sometimes fatal, Neisseria infections (18-19). This suggests that the

C5-8 complex can fulfill most of the functions of the membrane attack complex.

In attempting to characterize the mechanism of complement-mediated lysis, investigators have focused mainly on the C5-9 complex and, in particular, on the nature of the membrane lesion (i.e., detergent-effect or channel), the molecular events required for the formation of the membrane lesion, and the "pore" size of this lesion. Kinsky (10) proposed that complement induced membrane damage by a detergent-like mechanism. In fact, it has been shown that activation and deposition of a large dose of complement on the surface of E. coli resulted in the release of 60 to 70% of bacterial phospholipid (20, 21). Large doses of complement have also been shown to disintegrate the envelope membranes of murine leukemia viruses (22). However, evidence has accumulated that, at low doses, complement-mediated lysis does not involve a detergent-like mechanism, but rather induces the formation of channels. The dose response of complement lysis of red cells showed hyperbolic curves which Mayer interpreted as indicative of a one-hit mechanism in which one functional C5-9 complex or channel was sufficient to lyse the cell (23, 24). Mayer argued that if complement lysed the cell by a detergent-like mechanism, the process would be cooperative and would

result in sigmoidal dose response curves. Stronger evidence that structural channels are involved in complement-mediated lysis come from studies showing that (1) the sequential addition of the late complement components, C5b through C9, to one side of a black lipid membrane (single lipid bilayer that forms a barrier between two aqueous solutions) or to cells led to discrete conductance steps indicative of channel formation (25, 26); (2) membrane-bound C5-9 complexes were visualized as hollow, cylindrical structures by electron microscopy (27-29); (3) C5-8 and C5-9 possessed well-defined channel sizes as determined by molecular sieving studies on red blood cell ghosts using markers of different Stokes radii (30-34). The diameter of the C5-8 complex was found to be greater than 8.8 Å but less than 30 Å, while that of the C5-9 complex was found to be greater than 30 Å but less than 60 Å.

The formation of transmembrane complement channels has been found to involve the exposure of hydrophobic binding sites followed by the insertion of proteins into the membrane as the assembly process proceeds. Evidence for hydrophobic binding sites comes from the demonstration of the strong binding capacities of C5b-7, C5b-8 and C5b-9 complexes for detergents and phospholipids (35-38). Furthermore, charge-shift

electrophoresis experiments showed that C5b-6, C5b-7, C5b-8 and C5b-9 were amphiphilic while the individual, uncomplexed proteins were shown to be hydrophilic (35). Thus, these results indicated that the assembly of complement complexes on membranes involves conformational changes in the native hydrophilic proteins which expose hydrophobic lipid binding sites.

In addition to binding membrane phospholipids, it has been shown that the assembly of C5b-9 proteins involves the insertion of all of the terminal components into the lipid bilayer. Early studies in which membrane-bound complexes were treated with high salt and proteolytic enzymes (6, 39) provided indirect evidence that portions of these proteins inserted into the membrane during the formation of the C5-9 complex. Later studies using a membrane-restricted photoreactive probe which only labels intrinsic membrane proteins within the hydrophobic phase of the membrane, provided direct evidence that all the terminal components C5b through C9 proteins assembled and inserted into the membrane (40-42). Furthermore, the insertion of C8α and C9 could be correlated with red cell lysis (43, 44).

The transmembrane nature of complement channels was shown by experiments with RBC ghosts in which C9 and C8 molecules of the membrane-bound C5-9 complex

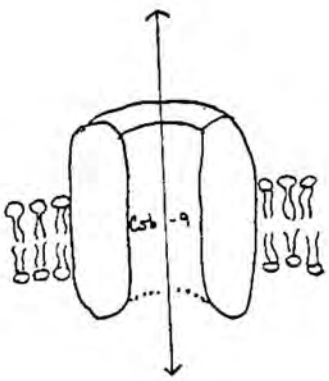
were enzymatically crosslinked on the cytoplasmic side of the membrane by red cell transglutaminase (45). Furthermore, both C9 and C8α were labeled by a photoprobe in both monolayers of the membrane (46).

Biochemical studies have shown that the C5-9 complex consists of one molecule each of C5b, C6, C7, and C8 (47, 48) and a variable number of C9 molecules (1-16) per C5-8 complex (47-49). The number of C9 molecules per C5-8 complex has been shown to depend on the temperature at which C9 is added to the C5-8 complex (50). Bhakdi has shown, that when C9 is added to RBC's bearing assembled C5-8 complexes at 4 °C, a $C5\text{-}9_1$ complex is formed in which an average of one C9 binds per C5-8 complex. In addition, molecular sieving and ultrastructural studies have shown that C5-9 complexes exhibit pore size heterogeneity (51-57), which has been attributed to the varying number of C9 molecules per C5-8 complex (52, 53).

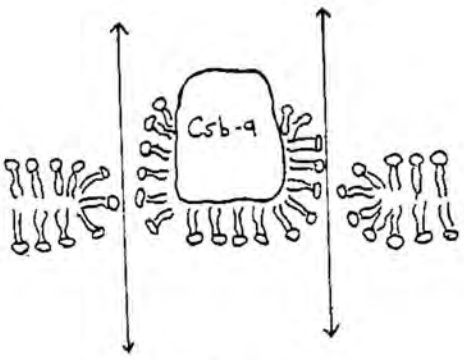
Investigators have attempted to describe the structure of the C5-9 complex responsible for membrane damage. Figure 2 shows four possible models for the complement lesion. Figure 2A describes a transmembrane, hollow, protein-walled channel as proposed by Mayer (doughnut hypothesis). Although the morphology of complement lesions on cells and liposomes as visualized by electron microscopy is consistent with

Figure 2

Models for the Membrane-Bound C5-9 Complex Showing the Location of the Putative Hydrophilic Channel: (A) Doughnut model, (B) Leaky patch or mixed micelle, (C) Lipid channel model, (D) Lipid-protein channel model. The complexes represented in (A), (C) and (D) are transmembrane while the complex shown in (B) does not span the membrane. Arrows denote the hydrophilic passageway.



A



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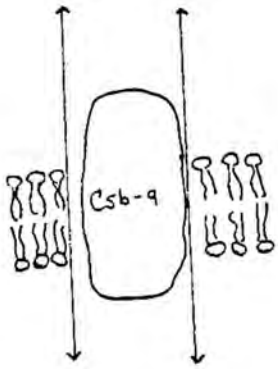

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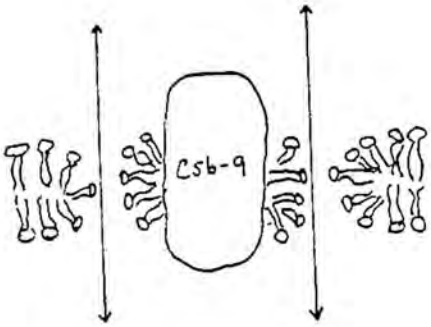


D

this structure (27-29), it has recently been shown that only one C9 molecule was necessary for hemolysis of RBC's (50). Podack et al. proposed the leaky patch or lipid-protein micelle model (Figure 2B) on the basis of the strong binding of complement complexes to phospholipid (36-38) and electron spin resonance studies on ordered lipid bilayers, in which C5-9 complexes induced lipid reorientation (58). This model, as proposed, is in conflict with data reporting direct evidence for the transmembrane nature of C5-9 since it does not view the C5b-9 complex as spanning the membrane. Other types of possible structures formed in the membrane are lipid channel or lipid-protein channel models in which C5-9 complexes disrupt the order of phospholipids in the membrane. With evidence that functional complement lesions could be formed by the association of C5-8 (59) or C5-9 complexes (57), one can envision that the aggregation of complexes might lead to the formation of lipid channels or lipid-protein channels (either Models C or D) resulting in membrane disruption and lysis.

Thus, considerable controversy still remains concerning the role of the number of C9 molecules per C5-8 complex and the predominant structure of the C5-9 complex on membranes. Similarly, the structure of the C5-8 complex is not clear and very little is known

about how C5-8 interacts with and affects the permeability properties of the membrane. There has also been much speculation about the role of the C5-8 complex in C5-9-mediated lysis. Three putative functions have been attributed to C5b-8 complexes relative to C5-9 assembly and function. First, C5-8 serves as a receptor for C9 (60). Second, C5b-8 destabilizes the target membrane to facilitate C9 insertion into the membrane (61). Third, the primary role of C5-8 is to catalyze C9 polymerization (61, 62) and, consequently, the formation of a large, stable channel. Despite these proposals, the relationship between the C5-8 and C5-9 complexes in terms of the nature of the respective channels formed is not known.

The primary objective of this project is to characterize the channel properties of the C5-8 complex in a model membrane system and to compare these properties with those of the better studied C5-9 complex. The interaction of C5-8 and C5-9 complexes with model membranes was investigated using large unilamellar vesicles (LUV's). As described above, vesicle systems (liposomes) composed of phospholipids and cholesterol can serve as targets for "reactive lysis" by the terminal complement components, C5-9, indicating that the lipid bilayer is the direct substrate for complement attack (11). In comparison to

natural targets, such as red blood cells (RBC's) and ghosts, LUV's provide several advantages for studying membrane-protein interactions. For example, membrane-complement interactions can be studied without the possible interference from intrinsic membrane proteins and the properties and composition of the lipid bilayer can be easily varied. With respect to other model systems, such as multilamellar vesicles (MLV's), LUV's have the advantage of having only one bilayer for the complement proteins to interact with. Small unilamellar vesicles (SUV's), on the other hand, tend to fuse upon addition of complement proteins and are relatively resistant to complement attack (70, 71). Thus as a membrane model, LUV's offer the advantages of a single, protein-free bilayer of adequate size and stability to study the interactions of complement complexes with membranes.

An assay which measures the release of the fluorescent dye, 6CF, from 6CF-loaded LUV's bearing C5-7 complexes was employed to determine the functional activity of C5-8 and C5-9 complexes after the addition of C8, or C8 and C9, to these vesicles. Vesicles loaded with entrapped markers have frequently been used to investigate membrane-protein interactions. Specifically, the release of 6CF from vesicles treated

with lytic agents has been used as evidence of channel formation or membrane perturbation (73). The 6CF release assay provided a convenient method to test conditions in optimizing C5-8 and C5-9 assembly on vesicles as well as to characterize C5-8 and C5-9 channel properties on vesicles.

It is the intent of these studies to determine the similarities and differences of the C5-8 and C5-9 complexes with respect to the size of the membrane lesion, channel stability, and the physicochemical factors affecting channel formation and function. It is hoped that such studies will better elucidate the relationship between the membrane-damaging C5-8 and C5-9 complexes.

EXPERIMENTAL PROCEDURES

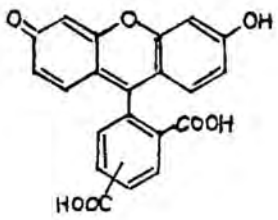
A. MATERIALS

The lipids, dimyristoyllecithin, cholesterol and dicetyl phosphate were purchased from Calbiochem, Applied Science Laboratories, and Sigma, respectively. 6-carboxyfluorescein (Figure 3A) was purchased from Eastman Kodak. Sephadex G-25 and Sepharose-4B were obtained from Pharmacia. [6,6'(n)-^{3}H]sucrose (spec. act. = 5-15 Ci/mmol), ^{3}H-inulin (spec. act. = 1-5 Ci/mmol), N-succinimidyl-[2,3,-^{3}H]propionate (spec. act. = 75-110 Ci/mmol), 1-palmitoyl-2-[9,10,(n)-^{3}H]-palmitoyl L-3-phosphatidylcholine (spec. act. = 30-60 Ci/mmol, and 1-palmitoyl-2-[1-^{14}C] palmitoyl-L-3-phosphatidylcholine (spec. act. = 40-60 nCi/mmol) were purchased from Amersham. Purified complement components C5, C6, C7, C8, and C9 were obtained from Cytotech. Purity of components is assessed by SDS-polyacrylamide electrophoresis. Goat antibodies to human complement were purchased from Miles while peroxidase-labeled rabbit anti-goat IgG was obtained from Kirkegaard & Perry. Antibodies are purified using solid phase immunoabsorbents. Purity is established

Figure 3

The structure of 6-carboxyfluorescein (A) and the glycolipid photoprobe, 12-(4-azido-2-nitrophenoxy)-stearoyl[1-^{14}C]glucosamine (B).

A



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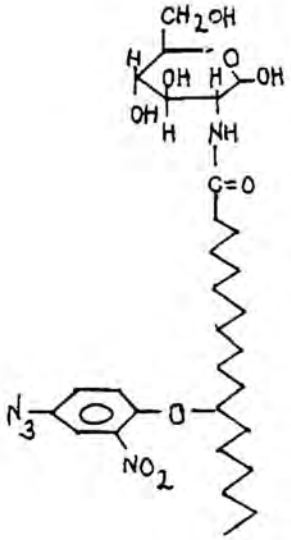


by immunodiffusion and immunoelectrophoresis. The photoprobe, 12-(4-azido-2-nitrophenoxy)stearoyl-[1-^{14}C]glucosamine (12-APS-GlcN, Figure 3B), was synthesized as previously described (40). Its specific activity was 278 mCi/mmol.

B. BUFFERS

PBS: 150 mM NaCl, 5 mM Na_2HPO_4 (pH 7.4)

K-PBS: 150 mM KCl, 5 mM K_2HPO_4 (pH 7.4)

TNB: 10 mM Tris, 0.5 M NaCl, 0.1% BRIJ (pH 7.4)

TBS: 46 mM Tris, 0.17 M NaCl (pH 8.0)

SDS Tris-glycine buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS

Electroblot buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol

C. METHODS

Preparation of LUV's by Reverse Phase Evaporation

LUV's, prepared by the reverse phase ether evaporation method (63) were composed of dimyristoyllecithin, cholesterol and dicetyl phosphate in a lipid molar ratio of 4:3:1 and contained entrapped 6-carboxyfluorescein (6CF) at a self-quenching (fluorescence) concentration of 100 mM, pH 7.4. The lipids were dried to a film by rotary evaporation under reduced pressure in a 50 ml round bottom flask and stored in a dessicator under vacuum overnight. 3 ml of ether was added to the lipid film which was then vortexed. One ml of 100 mM 6CF was added to the lipid-ether mixture and the solution was briefly sonicated with a tip sonicator until an emulsion was formed. The emulsion was placed on a rotary evaporator and the ether was removed under reduced pressure at 37 °C. During evaporation, the solution formed a gel which subsequently collapsed into an aqueous suspension. At this stage, an additional 2 ml of 6CF was added and the remaining ether was removed under higher vacuum. After all the ether was evaporated, the vesicles were passed through 0.4 and 0.2 micron nuclepore filters and then heated for 30 minutes at 37 °C to induce annealing.

After annealing, the vesicles were passed sequentially over Sephadex G-25 and Sepharose-4B columns (15-30 ml) in order to remove extravesicular 6CF. For most studies, the lipid mixture was doped with radiolabeled dipalmitoylphosphatidylcholine (spec. act. = 40-60 mCi/mmol) to facilitate the determination of lipid concentration throughout the experiments. It was assumed that the vesicles formed in 100 mM 6CF contained a concentration of entrapped dye of 100 mM.

Sizing of LUV's

LUV's were sized by negative-stain electron microscopy (EM) and light scattering. Vesicles were diluted to a concentration of 100 ug lipid/ml for electron microscopy. Vesicle samples were applied to Formvar-coated grids, negatively stained with 2% phosphotungstic acid, pH 7.4 and scanned using a Zeiss 10A Transmission Electron Microscope. Vesicle diameters were measured on photographs taken of the EM image. Magnification was 12500X. Three different vesicle preparations were analyzed by EM. Two hundred vesicles per preparation were sized. Vesicles diluted to 100 ug lipid/ml were also sized by light scattering methods using a NICOMP MODEL 370 submicron particle sizing system. Particle size was determined from fluctuations in the intensity of scattered light caused

by Brownian motion. The machine was calibrated with latex beads. Particle size distributions from 0.003 to 5 micrometers can be determined by the NICOMP Model 370.

Assembly of C5-8, C5-9 and C5-9_1 Complexes on Vesicles

The assembly of the C5-8 or C5-9 complexes on membranes involves interaction of 5 or 6 different components, including the membranes. To simplify the study of the processes involved in C5-8 and C5-9 channel formation and to avoid competing reactions involving the assembly of solution phase complexes, the pre-assembly of C5-7 complexes on membranes and the removal of unbound components prior to the addition of C8, or C8 and C9 was necessary. Once C5-6 associates with C7 on the membrane, the resulting complex becomes stable and does not dissociate from the membrane (72). Utilizing membranes bearing assembled C5-7 complexes simplified the assembly of C5-8 and C5-9 complexes to the interaction of solution phase C8, or C8 and C9 with membrane-bound C5-7. Figure 4 shows a flowchart of the assembly process and Figure 5 shows the competing membrane-bound and solution phase reactions of C5, C6 and C7.

The method of assembly of C5-8, C5-9 and C5-9_1 complexes on LUV's involved the acid-activation of C5

Figure 4

Flowchart of the preparation of 6CF-loaded LUV's bearing C5-7 complexes.

PROTOCOL FOR PREPARATION OF C5-7 VESICLES WITH ENTRAPPED 6CF

MIXTURE OF DML: CHOL: DCP (4:3:1) IN ETHER/ DYE EMULSION

↓

REVERSE PHASE EVAPORATION

↓

LARGE UNILAMELLAR VESICLES (LUV's)

↓

SEPHADEX G-25

↓ **REMOVAL OF UNENTRAPPED DYE**

SEPHAROSE-4B

↓

VESICLES **(WITH ENTRAPPED 6CF)**

↓ **+ (C5-6)AA , 10 ' ,37°C**

↓ **+ C7 , 5 ' , 37°C**

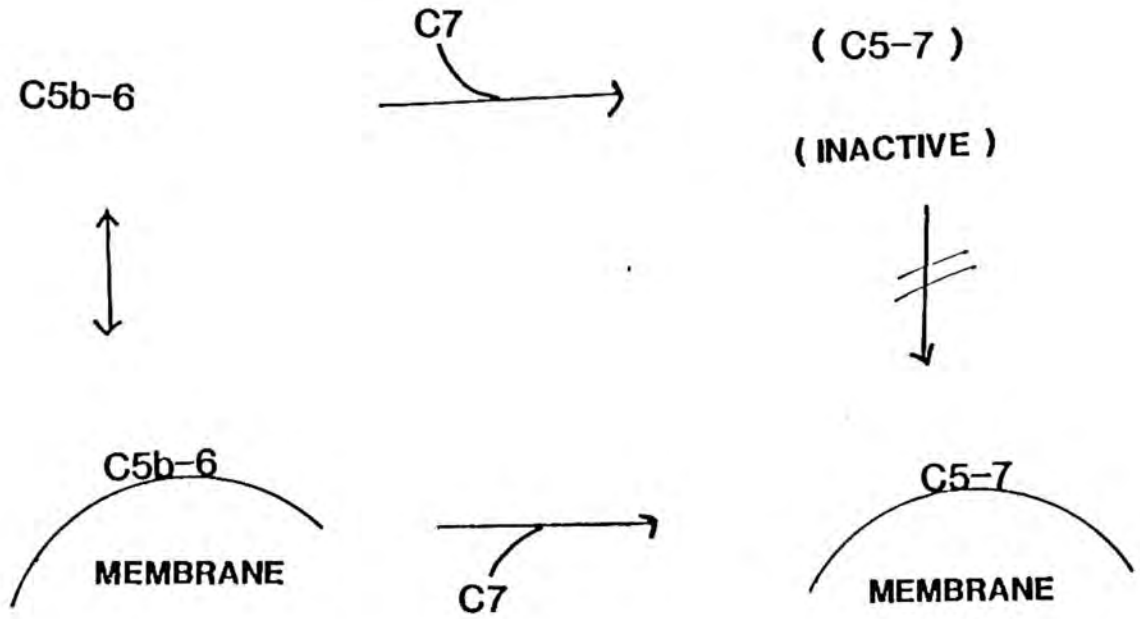
6ml SEPHAROSE 4B column

↓

C5-7 VESICLES

Figure 5

Competing solution phase and membrane-bound reactions leading to the formation of inactive and active C5-7 complexes, respectively.



together with C6 to form a C5b6-like complex (64). C5 and C6 were combined in phosphate buffered saline (PBS) at neutral pH and then exposed to a brief acid pulse (with 0.1 N HCl) to pH 5.5 at 4 °C. The pH was immediately returned after acid-activation to 7.0 with 0.1 N NaOH, and C5-6 was incubated with 6CF-loaded LUV's for 10 minutes at 37 °C, followed by a 5 minute incubation of C7 with C5-6 LUV's at 37 °C. The vesicles were rapidly separated from free, unbound proteins by passage over a 6 ml Sepharose-4B column which was centrifuged at 400 rpm for 15 seconds at 4 °C in a IEC centra-7R refrigerated table top centrifuge. The eluted vesicles possessed C5-7 on their surfaces and did not release dye. C5-8 and C5-9 complexes were generated by the respective addition of purified C8 or C8 and C9 to LUV's bearing C5-7. C5-9_1 complexes were assembled on 6CF-loaded LUV's according to the method of Bhakdi (50). C5-7 complexes were assembled as described above. 16 ug of C8 and 20 ug of C9 were added to 200 ug of C5-7 vesicle lipid at 4 °C and incubated for 1 hour at 4 °C. If Bhakdi's results with RBS's are valid for LUV's, then addition of C9 at 4 °C to vesicles bearing C5-8 complexes should result in C5-9 complexes having an average of one C9 bound per C5-8. Thus, these vesicles are nominally designated as "C5-9_1" LUV's. After incubation, LUV's bearing "C5-9_1"

complexes were separated from free proteins by passage over a 6 ml Sepharose column equilibrated at 4 °C and centrifuged at 400 rpm for 15 seconds at 4 °C. One half (0.5) ml vesicle fractions were eluted with ice-cold PBS.

Assay of the Functional Activity of C5-8, C5-9 and $C5\text{-}9_1$ Complexes

The functional activity of C5-8, C5-9, or $C5\text{-}9_1$ complexes on LUV's was determined by monitoring the release of entrapped 6CF from C5-7 LUV's after the addition of C8 or C8 and C9 to these vesicles. For most experiments, 6CF was entrapped in LUV's at a self-quenching concentration of 100 mM. Upon dye release and dilution in the extravesicular medium, the quenching was relieved and fluorescence of the dye immediately increased. The dequenching of dye permitted continuous monitoring of 6CF release from vesicles. An SLM 8000 spectrofluorometer was used to monitor fluorescence, with excitation at 490 nm and emission measured at 520 nm. The slit widths were adjusted to 2 nm. C5-8- mediated release was initiated by the addition of 3 ug of C8 to 2 ml of PBS containing 10 ug of C5-7 LUV lipid. The assay temperature was held constant at 37 °C by a Lauda RM 6 water circulator. Endpoint 6CF release was determined as the

time at which dye efflux ceased. The total releasable fluorescence was determined by the addition of 25 ul of 2% Triton X-100 to the vesicle samples. Similarly, C5-9-mediated dye release was initiated by the addition of 3 ug of C8 and 3 ug of C9 to 10 ug C5-7 vesicle lipid in 2 ml PBS at 37 °C and 6CF release was followed to endpoint. For C5-9_1 samples, aliquots of C5-9_1 vesicles at 4 °C were added to 2 ml of PBS at 37 °C and monitored fluorometrically for 6CF release. The amount of dye released from these vesicles was compared to the amount released from vesicles on which C8 and C9 were assembled at 37 °C. Complement activity on vesicles was assessed by determining:

Percent 6CF Release: $(F-Fo)/(Ft-Fo) \times 100$

where F = endpoint fluorescence after addition of C8 or C8 & C9 to C5-7 LUV's

Fo = initial fluorescence of C5-7 LUV's

Ft = total fluorescence after Triton-x-100 addition

Rate of 6CF Release: $\Delta F/\Delta t$

Where F = change in fluorescence

t = change in time (seconds)

The rate of 6CF release was determined from the slope of the line hand drawn tangentially to the steepest portion of the dye release curve.

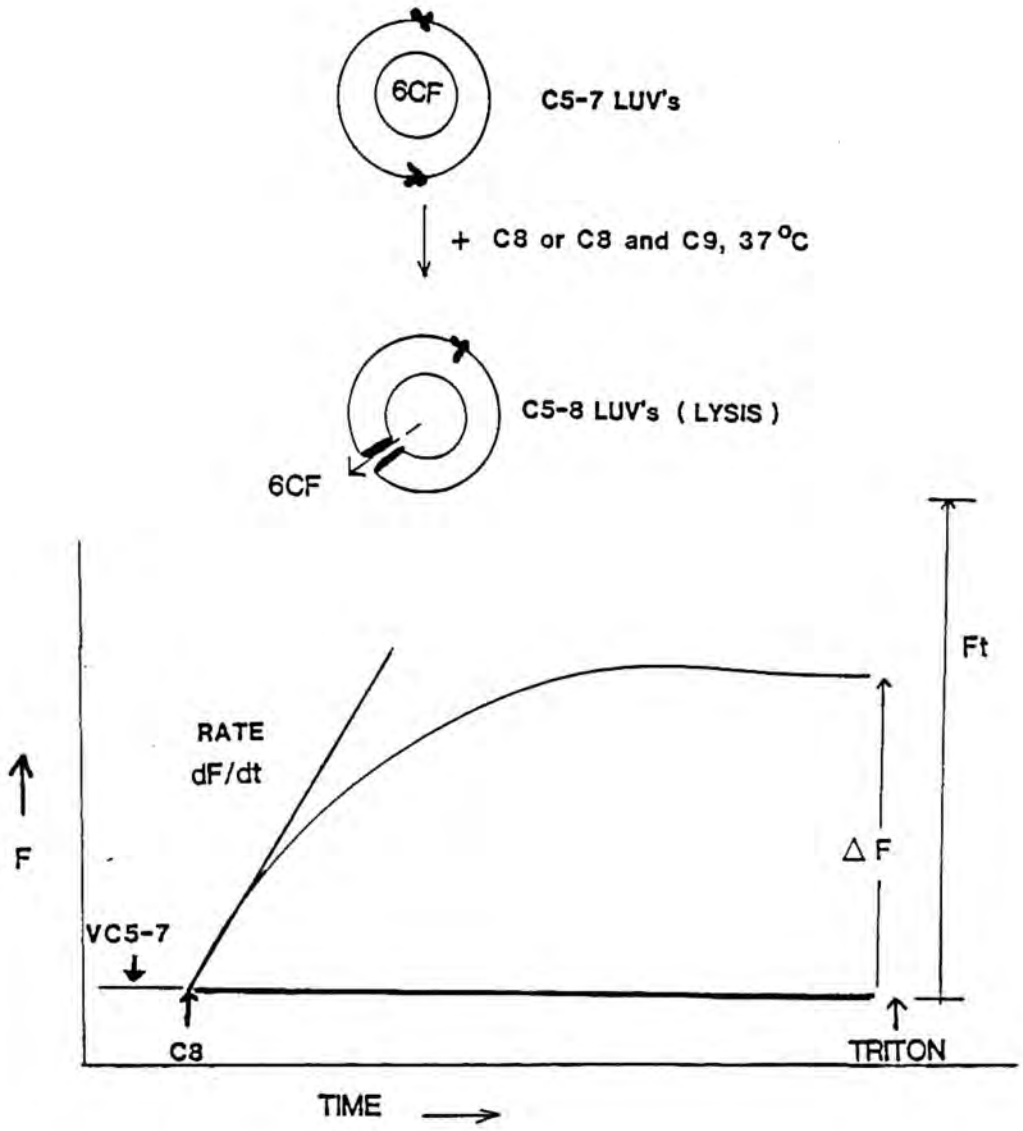
A schematic representation of the 6CF release assay is shown in Figure 6.

Determination of the Mode of 6CF Release from LUV's

The mode of 6CF release from LUV'S bearing C5-8, C5-9, or C5-9_1 complexes was assessed to determine whether vesicles bearing a complement lesion released their total content (all-or-none release), or only a portion of entrapped dye (graded release). The assessment was possible because the fluorescence of entrapped dye was highly quenched at 100 mM 6CF. Partial (graded) release of dye from LUV's results in the dilution of dye within vesicles; quenching is partially relieved and the intrinsic fluorescence within LUV's increases. Graded release could be differentiated from all-or-none release by determining the residual fluorescence within LUV's in the absence and presence of Triton. Fo/Ft ratios were measured and compared for C5-7, C5-8, C5-9 and C5-9_1 LUV's:

Figure 6

Assay for C5-8 and C5-9 Functional Activity: C8 or C8 and C9 were added to LUV's loaded with 100 mM 6CF bearing pre-assembled C5-7 complexes on their surfaces. The release of 6CF after C8 or C8 and C9 addition was followed to endpoint fluorometrically as a function of time. Total releasable dye was determined by the addition of 2% Triton X-100 to the vesicles. The % 6CF release was calculated as the maximum C8- or C8 + C9-induced change in fluorescence divided by the total releasable fluorescence, multiplied by 100. The rate of 6CF release was determined by the slope of the line drawn tangentially to the rapidly rising portion of the 6CF release curve. C5-8 and C5-9 complex activity was assessed by the percent and rate of dye release from complement-treated LUV's.



% LYSIS=ΔF/Ft x 100

Fo/Ft: Fo = residual LUV fluorescence after removal released dye by Sepharose-4B chromatography

Ft = total fluorescence after treatment of vesicles with Triton X-100.

If a fraction of complement-treated LUV's leaked all of their dye (all-or-none), then the Fo/Ft ratio of the remaining unlysed vesicles would be the same as the Fo/Ft ratio of the C5-7 control LUV's. However, if all complement-treated vesicles leaked only some of their dye, then the Fo/Ft ratio would be greater than the control. The increase in Fo/Ft would be determined by the percentage of entrapped dye released. Fo/Ft ratios of complement treated LUV's were compared to a quenching curve (made as described below) for standard vesicle preparations containing different concentrations of entrapped dye. The standard curve allowed the prediction of Fo/Ft values for graded release based on the percent of 6CF release.

The assay was performed as follows. C5-7 complexes were assembled as described in Materials and Methods and 10 ug (lipid) C5-7 LUV's in 2ml PBS were treated with 3 ug C8 or 3 ug C8 and C9. Release of 6CF was followed to endpoint fluorometrically. Aliquots (1 ml) of C5-7, C5-8 and C5-9 were passed over 6 ml

Sepharose-4B columns spun at 400 rpm for 15 secs at 4 °C to separate free 6CF from vesicles. Aliquots of the eluted vesicle fractions (0.5 ml) were analyzed for residual fluoresence in the absence and presence of Triton in order to determine Fo/Ft.

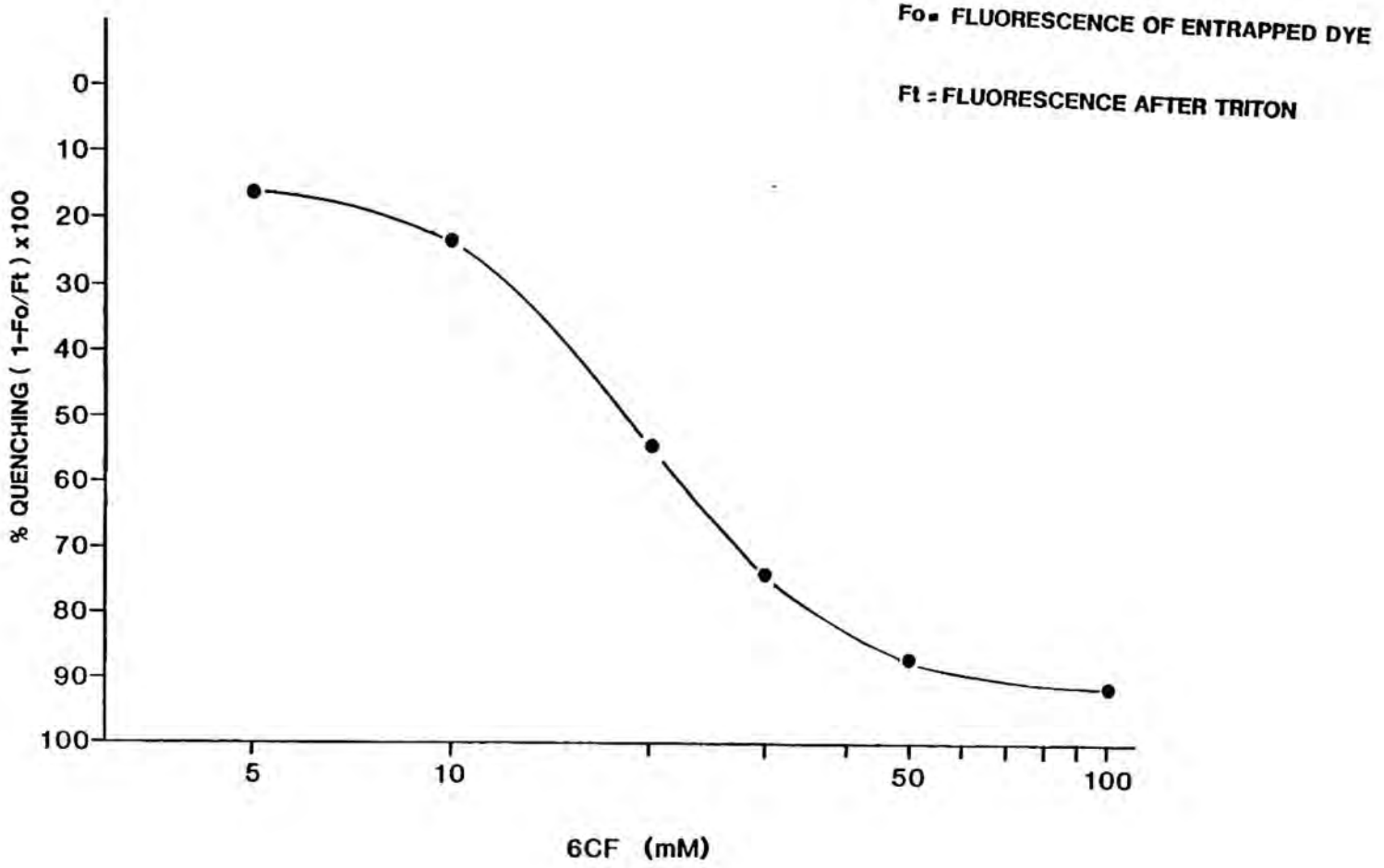
To establish a standard quenching curve, vesicles containing various concentrations (5-100 mM) of entrapped 6CF were prepared by reverse phase evaporation. Immediately after preparation, the Fo/Ft ratios of these vesicles were determined. A curve (Fig. 7) was made of % quenching vs. log of the concentration of entrapped dye. This curve was used to predict Fo/Ft ratios for any given percentage of 6CF release if a graded release mechanism were involved (65). For example, according to the curve, vesicles initially loaded with 50 mM 6CF (Fo/Ft = 0.13) will exhibit a higher Fo/Ft value of 0.36 corresponding to 25 mM trapped 6CF for 50% dye release.

C7 Binding Assay

^{3}H-C7 was used to determine the amount of C5-7 complexes assembled on LUV's. C7 was derivatized with N-succinimidyl-[2,3,-^{3}H]proprionate (^{3}H-NSP) (spec. act. = 107 Ci/mmol) according to the procedure of Muller (66). One hundred uCi of ^{3}H-NSP was dried down under nitrogen. One hundred ug of purified C7 in PBS (100 ul, pH 7.4) was added and the mixture was

Figure 7

Quenching Curve for 6CF Entrapped Within LUV's:
LUV's containing different concentrations of trapped 6CF were prepared by the reverse phase evaporation method. Immediately after vesicle preparation, the initial quenched fluorescence (Fo) and the total fluorescence (Ft), measured after the addition of Triton X-100 to LUV's, were determined for each sample. A quenching curve was made by plotting the percent quenching (1- Fo/Ft X 100) vs the log of the concentration of 6CF trapped within vesicles.


Fo = FLUORESCENCE OF ENTRAPPED DYE
Ft = FLUORESCENCE AFTER TRITON
% QUENCHING (1-Fo/Ft) x100
0
10
20
30
40
50
60
70
80
90
100
5
10
50
100
6CF (mM)

incubated for 36 minutes at 4 °C with constant stirring. Labeled protein was separated from free ^{3}H-NSP by a Sephadex G-25 column using PBS, pH 7.4, as the eluant. The column was pre-saturated with a 1% BSA solution in PBS to prevent non-specific adherence of protein to the column. Fractions (0.5 ml) containing ^{3}H-C7 were collected and stored at -70 °C until used. The amount of ^{3}H C7 recovered was determined by a micro-Lowry assay (67). For binding assays, 1-2 ug of ^{3}H-C7 was incubated for 5 minutes at 37 °C with 100 ug of 6CF-loaded LUV's bearing acid-activated C5-6. 1-2 ug of ^{3}H-C7 was also incubated under the same conditions with 100 ug lipid of LUV's alone to determine the amount of non-specific binding. To separate vesicles from unbound proteins, the vesicles were passed over 6 ml Sepharose-4B columns spun at 400 rpm for 15 secs at 4 °C. 250 ul aliquots of column fractions were added to 10 ml of Ready-Solv scintillation fluid and were counted in a Packard Scintillation counter. The amount of specifically bound C7 was determined after correction for non-specific binding.

Photolabeling Assay to Measure Insertion of Complement Proteins Into LUV's

Insertion of proteins into the lipid bilayer of LUV's was measured using the photoreactive probe, 12-(4-azido-2-nitrophenoxy)stearoyl[1-^{14}C]glucosamine (12-APS-GlcN). Probe (160,000 cpm in ethanol) was dried down to a small volume (1-2 ul) to keep the volume of ethanol below 1% of the total reaction volume. At this concentration, ethanol has no effect on bilayer structure. Two hundred ug of vesicle lipid were added and the solution was incubated at 37 °C for 15 minutes. C5-7, C5-8, or C5-9 complexes were then assembled on vesicles containing embedded probe according to methods described. Following assembly, LUV samples were irradiated at 366 nm for 10 seconds with a high power Blak-ray UV lamp held at a distance of one inch from the sample. The lamp produces an average intensity at 366 nm of 7000 uw/cm^2 at 38 cm. The vesicles were then harvested by centrifugation at 180,000 x g for 30 minutes. Vesicle pellets were solubilized in 25 ul of non-reducing SDS sample buffer (69) before application to polyacrylamide gels.

Electrophoresis and Electroblotting Procedures

7.5% polyacrylamide slab gels (40 ml) were prepared according to the method of Laemmli (69) with a 2.5% acrylamide stacking gel. Samples were solubilized in reducing sample buffer containing 8 M urea, boiled for 2 minutes and applied to gels. Electrophoresis was run in a 0.1% SDS/Tris/Glycine buffer at constant amperage (30 mA) for 3 to 4 hours. After electrophoresis, samples were electrophoretically transferred to nitrocellulose paper using a Hoefer transphor electroblot system. Nitrocellulose paper was applied to gels in a standard electroblot cassette. The cassette was immersed in electroblot buffer for 2 hours at 1 amp followed by overnight transfer at 0.3 amp. The resulting blots of photolabeled samples were stained with amido black to visualize the proteins. The blots were then dried and exposed to Kodak X-O-MAT AR film. The film was exposed for 2 weeks at room temperature. Quantitation of the relative amounts of inserted protein was achieved using a Hoefer GS-300 scanning densitometer. Autoradiograms were scanned in the transmittance mode. The paper corresponding to the areas under the densitometric peaks were cut out and weighed. Alternatively, the relative amounts of inserted proteins were determined by cutting out the protein bands from the stained electroblots and

determining the amount of ^{14}C counts associated with each band after immersion in Beckman Ready-Solv scintillation fluid. A Packard Tri-Carb Scintillation spectrometer was used.

Immunostaining of Electroblots

In some experiments, the protein bands in the electroblot were stained with antibodies against particular complement proteins. Blots were treated sequentially with goat anti-complement antisera (1:100 dilution) for two hours, peroxidase-labeled rabbit anti-goat IgG antiserum (1:1000 dilution) for 1 hour and finally a benzidine solution (50 mg/ml TBS) for 15 to 30 minutes. All incubations were done at room temperature with rocking. Between successive treatments, the blots were washed several times with TNB buffer. Quantitation of the protein bands was determined densitometrically as described above.

Detection of Ion Channels in LUV's Induced by Gramicidin

Ion channels induced by gramicidin addition to LUV's were detected by the change in fluorescence of the membrane potential-sensitive dye, diS-C_3-(5). The induction of a membrane potential results in a change in fluorescence of diS-C_3-(5) which is embedded in the

membrane (68). The fluorescence of diS-C_3-(5) was monitored as a function of time on a SLM 8000 spectrofluorometer with excitation at 622 nm and emission measured at 670 nm. The slit widths were set at 2 nm. One ug of diS-C_3-(5) was added to 300 ug of LUV lipid (containing entrapped 6CF, Na salt) in 2 ml of K-PBS (150 mM KCl, 5 mM K_2PO_4). After the fluorescence of diS-C_3-(5) had stabilized, a membrane potential (depolarization) was induced by the addition of 2 ug of gramicidin and the fluorescence change of diS-C_3-(5) was monitored. The magnitude of the membrane potential was controlled by the relative concentrations of K^+ and Na^+ in the extravesicular and intravesicular medium. In some experiments, both diS-C_3-(5) and 6CF fluorescence was monitored after LUV's were treated with gramicidin.

Determination of the "Pore Size" of the C5-8 and C5-9 Complexes (Molecular sieving experiments)

LUV's were prepared by reverse phase evaporation as described above with the modification that either ^{3}H-sucrose (spec. act. = 6 Ci/mmol) or ^{3}H-inulin (spec. act. = 5 Ci/mmol) was entrapped inside LUV's together with 6CF. One hundred and twenty-five uCi of one sugar or the other and 1 ml of 100 mM 6 CF were added to 3 ml of the ether-lipid mixture.

Vesicles were prepared as usual and separated from free 6CF and radioactive sugars by chromatography over 14 ml Sepharose-4B columns. Encapsulation efficiencies were determined by measuring the ratio of the amount of dye or sugars associated with vesicle fractions to the total (encapsulated and free) amount of these materials available for encapsulation.

Molecular sieving experiments were performed by assembling C5-8 or C5-9 complexes on LUV's loaded with both 6CF and sucrose or inulin. Three ug of C8 or 3 ug of C8 and C9 were added to 2 ml of PBS containing 10 ug lipid of C5-7 LUV's. The percent of 6CF release from vesicles was determined. One ml of each vesicle sample was applied to 6 ml Sepharose columns. Vesicle fractions (0.5 ml) were eluted from the columns by centrifugation at 400 rpm for 15 secs at 4 °C. Aliquots (200 ul) of vesicle fractions were added to scintillation vials containing 10 ml of scintillation fluid and counted in a Packard scintillation counter. ^{3}H counts associated with C5-7-, C5-8-, and C5-9-treated vesicles were measured. The percent release of radioactive sucrose and inulin from C5-8- or C5-9-treated LUV's was determined by comparing the residual trapped ^{3}H-marker associated with vesicle fractions with that of the C5-7 control after removal of released ^{3}H-sugars by gel chromatography.

Analysis of Complement-Treated LUV's by the Meridian ACAS 470 Fluorescence Workstation to Assess Susceptibility of Vesicles of Different Sizes to Complement

Suspensions of LUV's and complement-treated LUV's containing entrapped 6CF were applied to glass slides coated with 0.025% poly-L-lysine which served to anchor the vesicles. The glass slides were examined under a 100x oil immersion objective using the Meridian ACAS 470 Fluorescence Workstation. The workstation consists of a 2 watt argon ion laser light source, an Olympus IMT-2 inverted microscope, a Hamamatsu R 1547 photomultiplier tube and an IBM AT-compatible computer for data analysis. The instrument was used to visualize 6CF loaded LUV's and characterize dye release from complement-treated vesicles. Specific instrumental parameters for each measurement are provided in the appropriate figure legends.

RESULTS

I. Characterization of the Model System

Size Heterogeneity and Entrapment Efficiency of Reverse Phase LUV's

LUV's prepared by reverse phase evaporation were heterogeneous in vesicle size (Figure 8). Size estimates obtained by negative-stain EM showed that the vesicles ranged in size from 500-11000 Å with a mean vesicle size of 2500 Å ± 115 Å (Figure 9). This was corroborated by particle size data obtained by light scattering (Figure 10). The encapsulation efficiencies of these LUV's were 40-50%, 10-23%, and 10-33% for 6CF, sucrose and inulin, respectively, as shown in Figures 11, 12, and 13. These efficiencies compared well with reported encapsulation efficiences (25-60%) of vesicles prepared by reverse phase evaporation methods (63).

Assembly of Functional C5-8 and C5-9 complexes on LUV's

C5-7 was assembled on 6CF-loaded LUV's as described in Materials and Methods. Vesicles bearing these C5-7 complexes on their surfaces did not usually release dye when monitored fluorometrically.

Figure 8

6CF-loaded LUV's as visualized by negative-stain (phosphotungstic acid) electron microscopy. The bar indicates 325 nm.

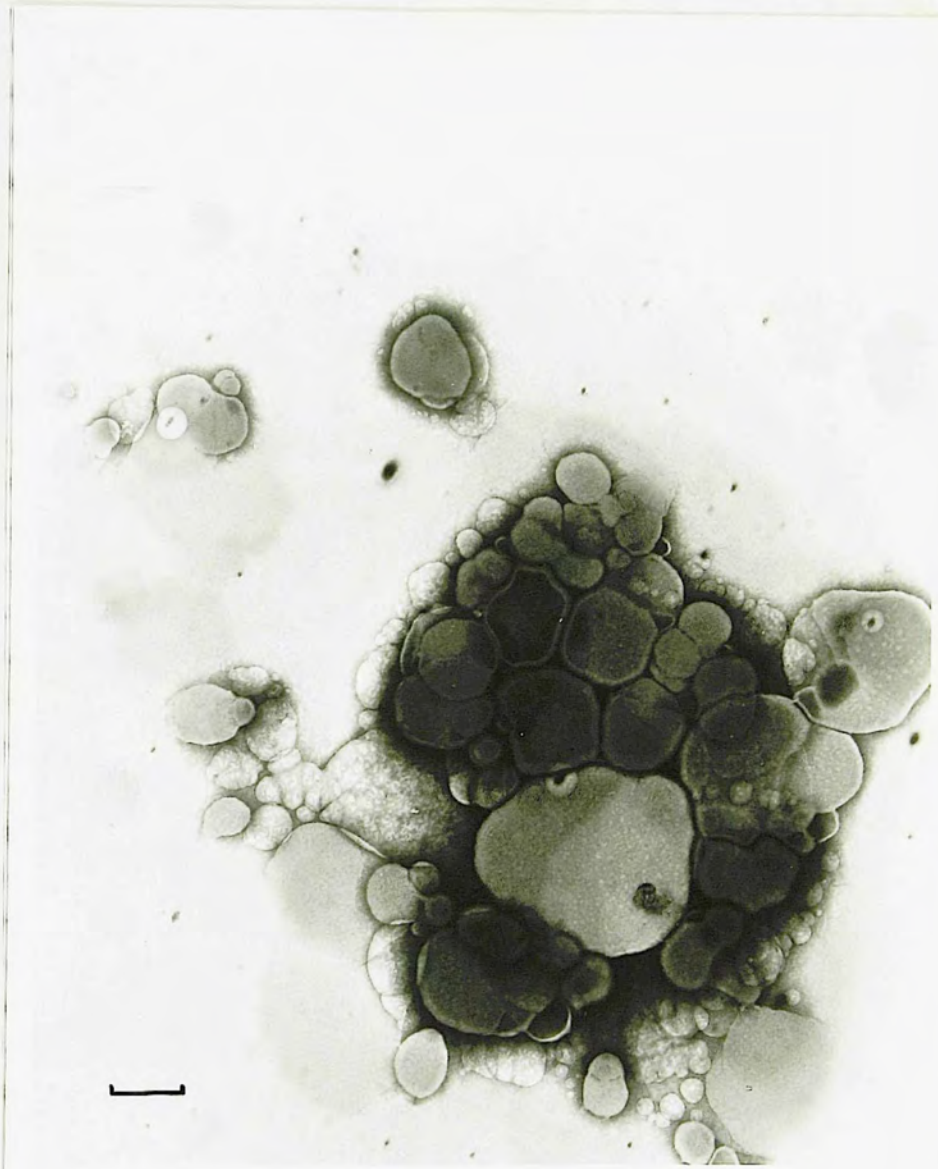


Figure 9

Size Distribution of 6CF-Loaded LUV's: The histogram shows the size distribution of 6CF-loaded LUV's as determined by negative-stain (phosphotungstic acid) electron microscopy. Vesicle diameters were measured on photographs of the images. The histogram represents data obtained from three separate vesicle preparations. A total of 200 vesicles per preparation were analyzed.

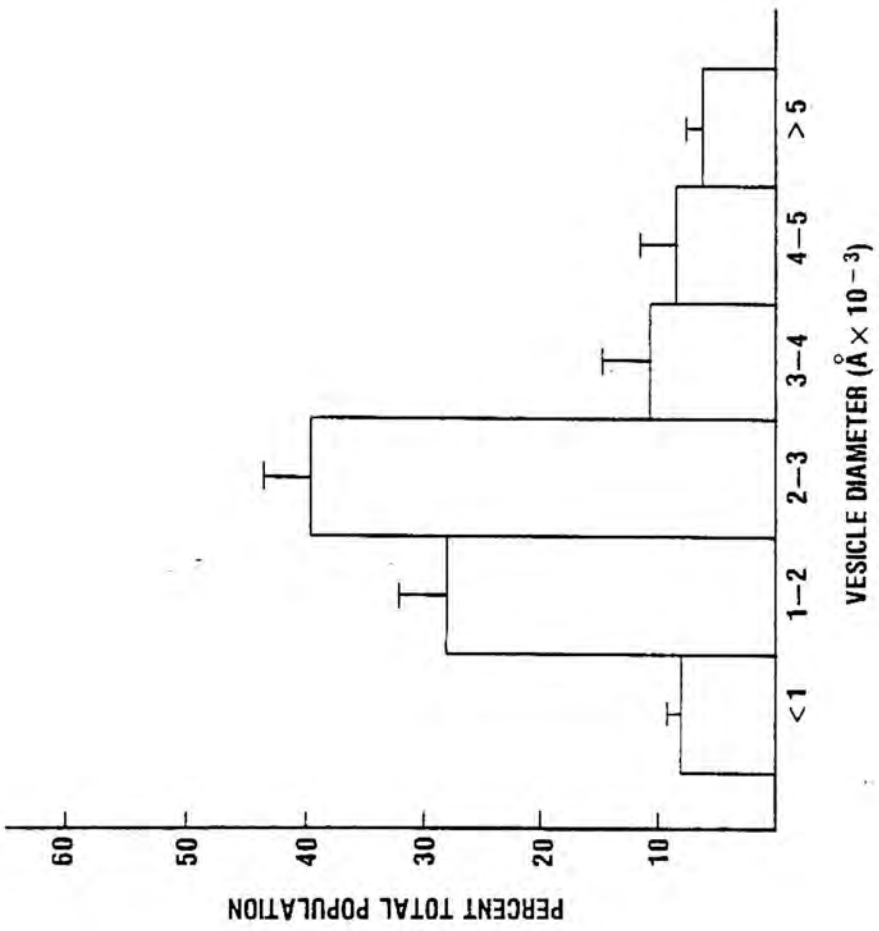

PERCENT TOTAL POPULATION
60
50
40
30
20
10
<1
1—2
2—3
3—4
4—5
>5
VESICLE DIAMETER (Å × 10⁻³)

Figure 10

Size Distribution of 6CF-Loaded LUV's: The histogram shows the size distribution of 6CF-loaded LUV's as determined by light scattering. Vesicle diameters were measured on a NICOMP Model 370 particle sizing system.

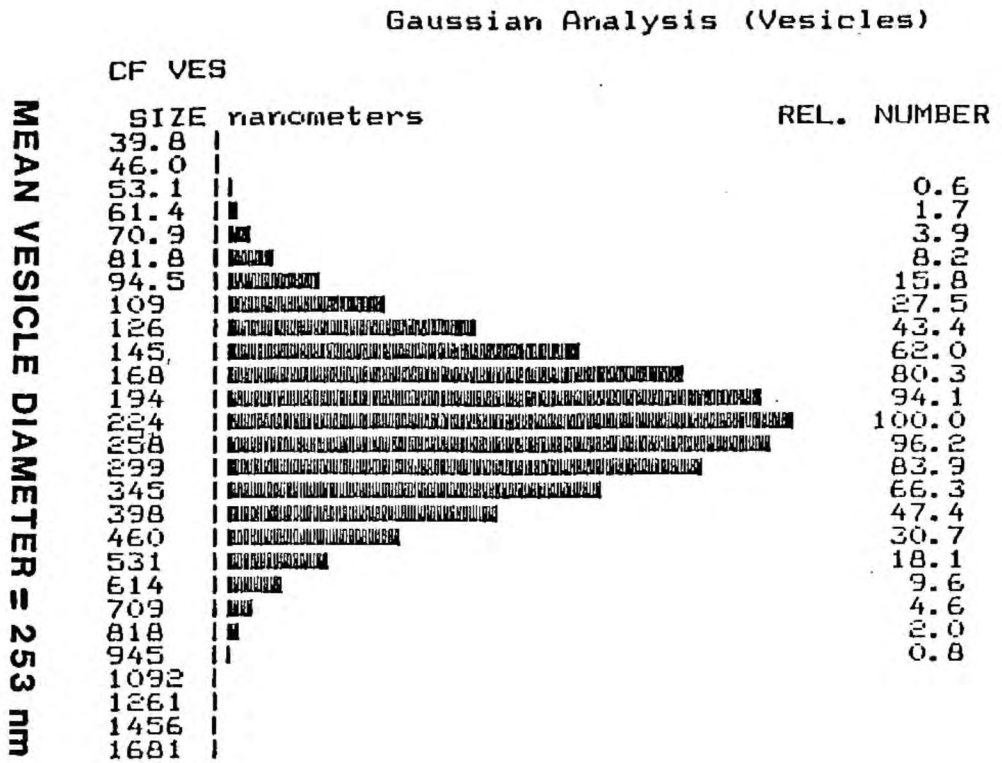

Gaussian Analysis (Vesicles)
CF VES
SIZE nanometers
REL. NUMBER
39.8
46.0
53.1
61.4
70.9
81.8
94.5
109
126
145
168
194
224
258
299
345
398
460
531
614
709
818
945
1092
1261
1456
1681
0.6
1.7
3.9
8.2
15.8
27.5
43.4
62.0
80.3
94.1
100.0
96.2
83.9
66.3
47.4
30.7
18.1
9.6
4.6
2.0
0.8
MEAN VESICLE DIAMETER = 253 nm

Figure 11

Separation of Free 6CF From LUV's: One hundred mM 6CF was encapsulated within LUV's by reverse phase evaporation. One ml of LUV's was run over a Sepharose-4B column (33 ml) with a flow rate of 1 ml/minute at 22 °C. One ml fractions were collected. Efficiency of 6CF encapsulation by these vesicles was 44%. LUV lipid concentration was determined by ^{14}C-dipalmitoylphosphatidylcholine.

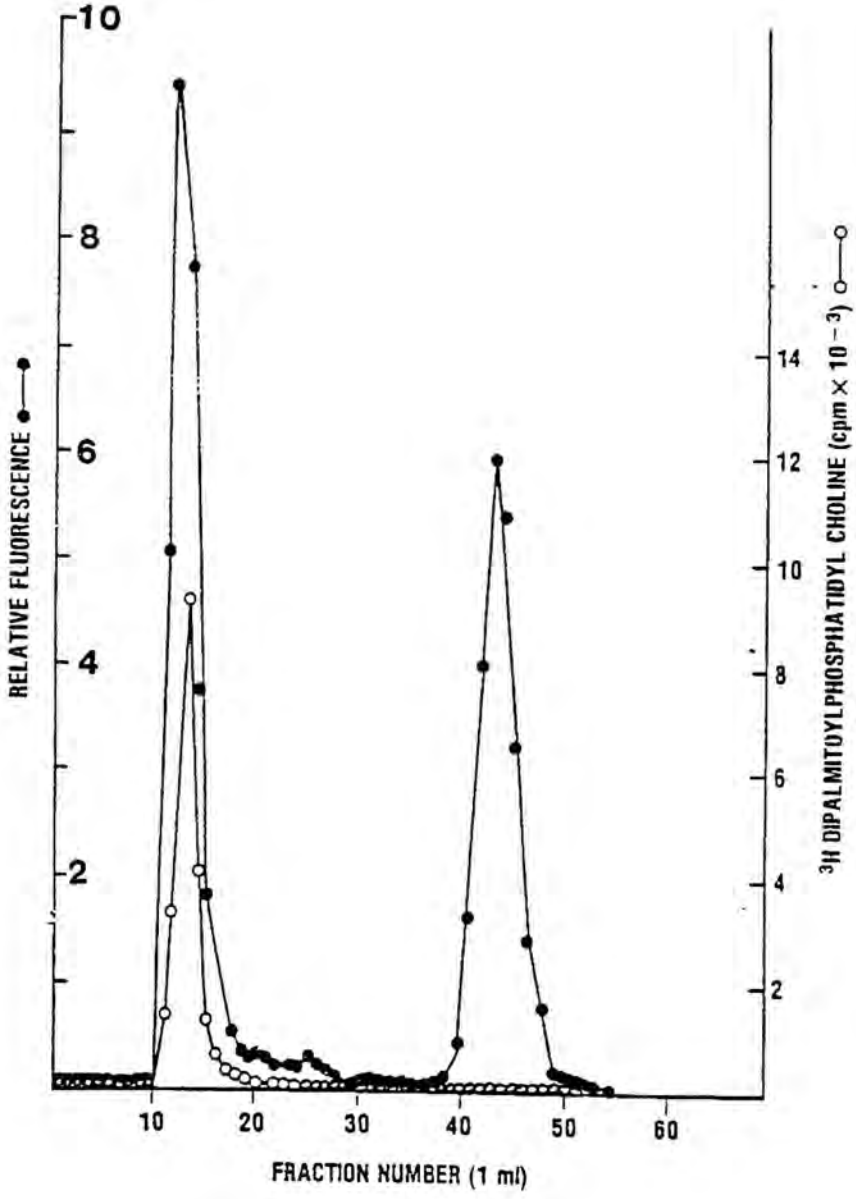

RELATIVE FLUORESCENCE
10
8
6
4
2
^{3}H DIPALMITOYLPHOSPHATIDYL CHOLINE (cpm × 10 – 3)
14
12
10
8
6
4
2
10
20
30
40
50
60
FRACTION NUMBER (1 ml)

Figure 12

Separation of Free ^{3}H-Sucrose From LUV's: ^{3}H-sucrose and 100 mM 6CF were entrapped within LUV's by reverse phase evaporation. One ml of LUV's were run over a Sepharose-4B column (14 ml) with a flow rate of 1 ml/minute at 22 °C. One ml fractions were collected. Efficiency of sucrose encapsulation by these vesicles was 23%. LUV lipid concentration determined by ^{14}C-dipalmitoylphosphatidylcholine.

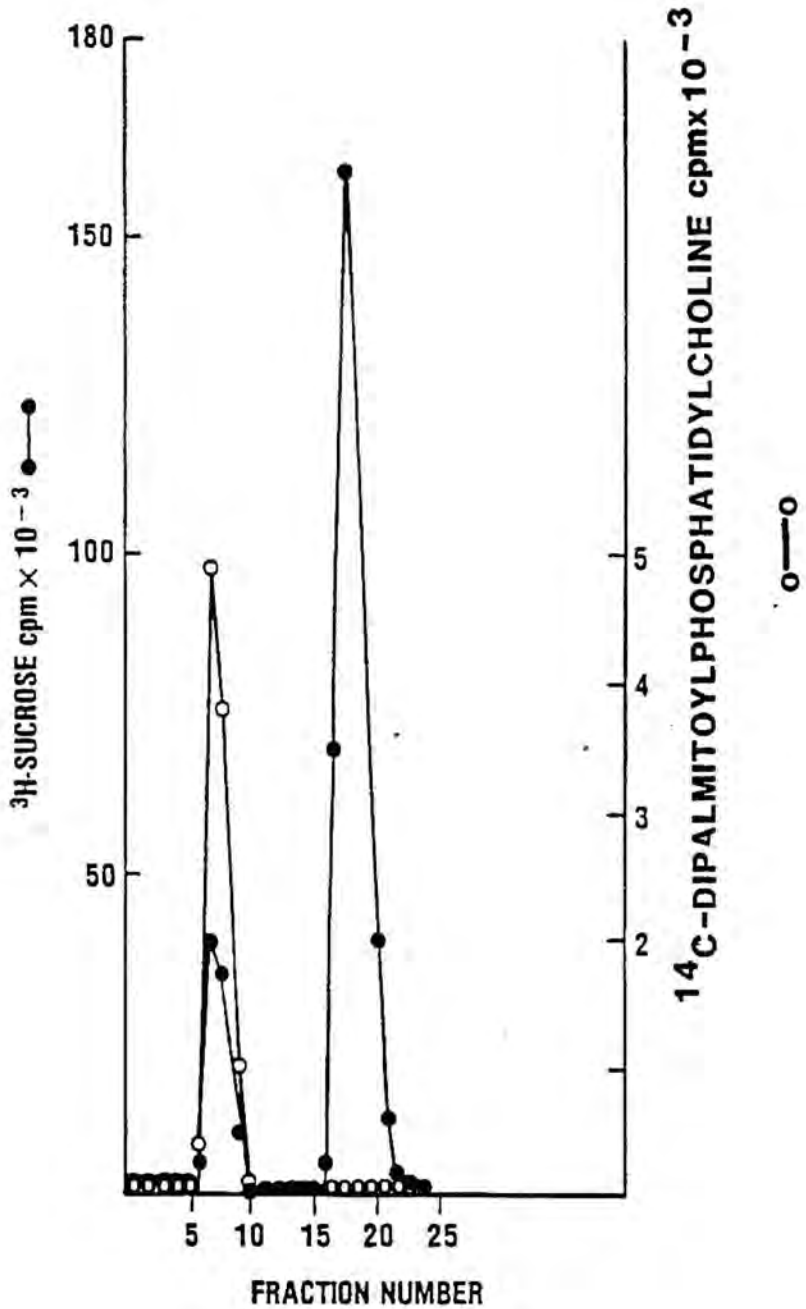
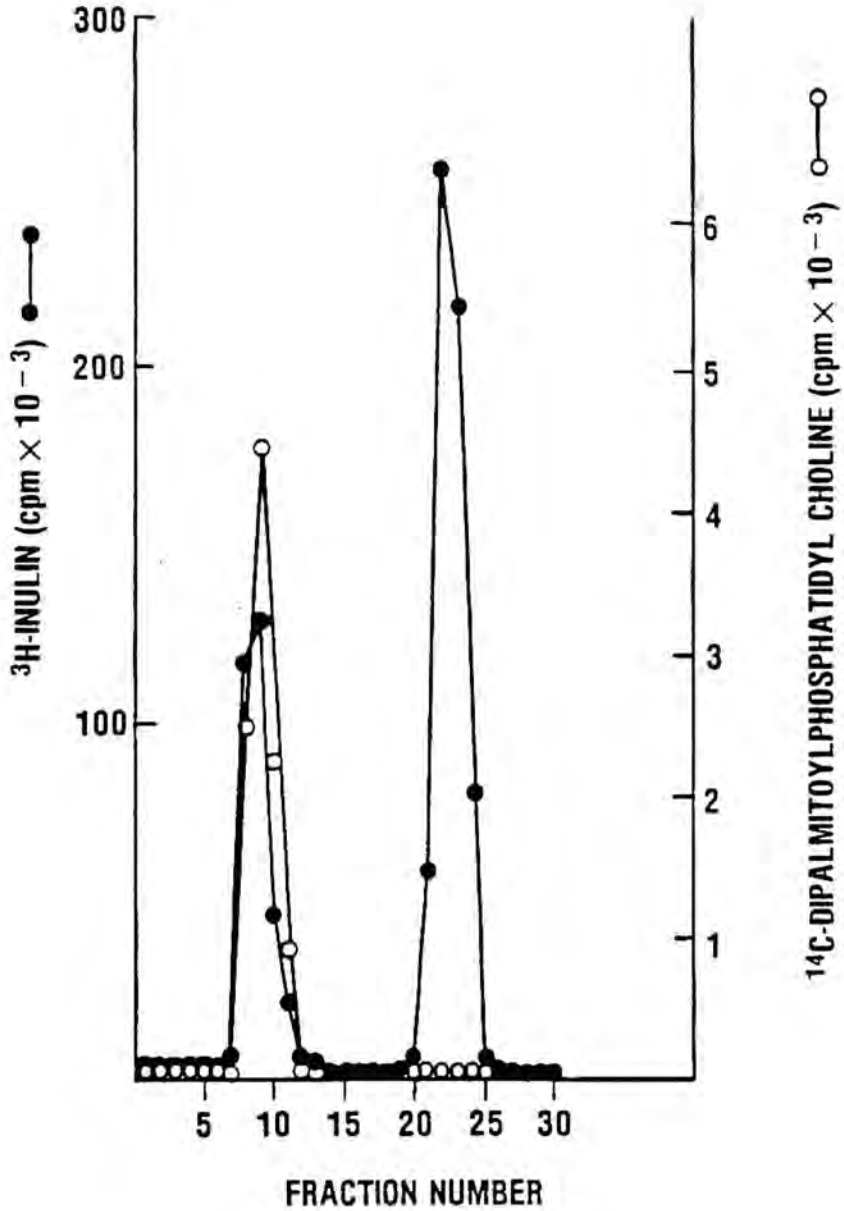

180
150
100
50
5
4
3
2
5
10
15
20
25
FRACTION NUMBER
^{3}H-SUCROSE cpm × 10^{-3}
^{14}C-DIPALMITOYLPHOSPHATIDYLCHOLINE cpmx10^{-3}

Figure 13

Separation of Free ^{3}H-Inulin From LUV's: ^{3}H-inulin and 6CF were entrapped within LUV's by reverse phase evaporation. One ml of LUV's was run over a Sepharose-4B column (14 ml) with a flow rate of 1 ml/minute at 22 °C. One ml fractions were collected. The efficiency of inulin encapsulation by these vesicles was 33%. LUV lipid concentrations was determined by ^{14}C-dipalmitoylphosphatidylcholine.


300
200
100
^{3}H-INULIN (cpm × 10 − 3)
6
5
4
3
2
1
^{14}C-DIPALMITOYLPHOSPHATIDYL CHOLINE (cpm × 10 − 3)
5
10
15
20
25
30
FRACTION NUMBER

Furthermore, addition of either C8 or C9 to 6CF-loaded LUV's alone did not cause the release of dye (Figure 14A), indicating that dye release required the assembly of the entire C5-8, or C5-9, complex on vesicles (Figures 14, B and C). To eliminate the possible complications caused by the presence of soluble C5-7 complexes, C5-7 LUV's were separated from unreacted proteins and solution-phase protein complexes by Sepharose-4B chromatography. This separation was demonstrated by the elution profile of C5-7 LUVs and ^{3}H-C7 on 6 ml Sepharose columns under low speed centrifugation (Figure 15). Vesicles bearing tritiated C5-7 complexes eluted well ahead of free C7. Additional evidence for separation of free proteins from C5-7 LUV's was provided by the functional 6CF release assay. In cases where 6CF release was less than 100%, it was possible to increase 6CF release by the addition of more acid-activated C5-6 and C7 in the presence of excess C8 and C9 (Figure 16). Addition of either acid-activated C5-6 alone, or C7 alone, did not cause the release of additional dye, thus demonstrating that there was no residual, unbound C5, C6, or C7 proteins in C5-7 LUV preparations after the minicolumn separation step. Furthermore, the fact that 6CF release could be increased by the successive addition

Figure 14

Release of 6CF From LUV's and C5-7 LUV's Treated With C8 and C9: LUV's contained 100 mM 6CF. C5-7 was assembled on 6CF loaded-LUV's as described in Materials and Methods. (A) control LUV's treated with C8 and C9. 6CF release from C5-7 LUV's treated with C8 (B) or C8 and C9 (C). Dye release was measured at 37 °C.

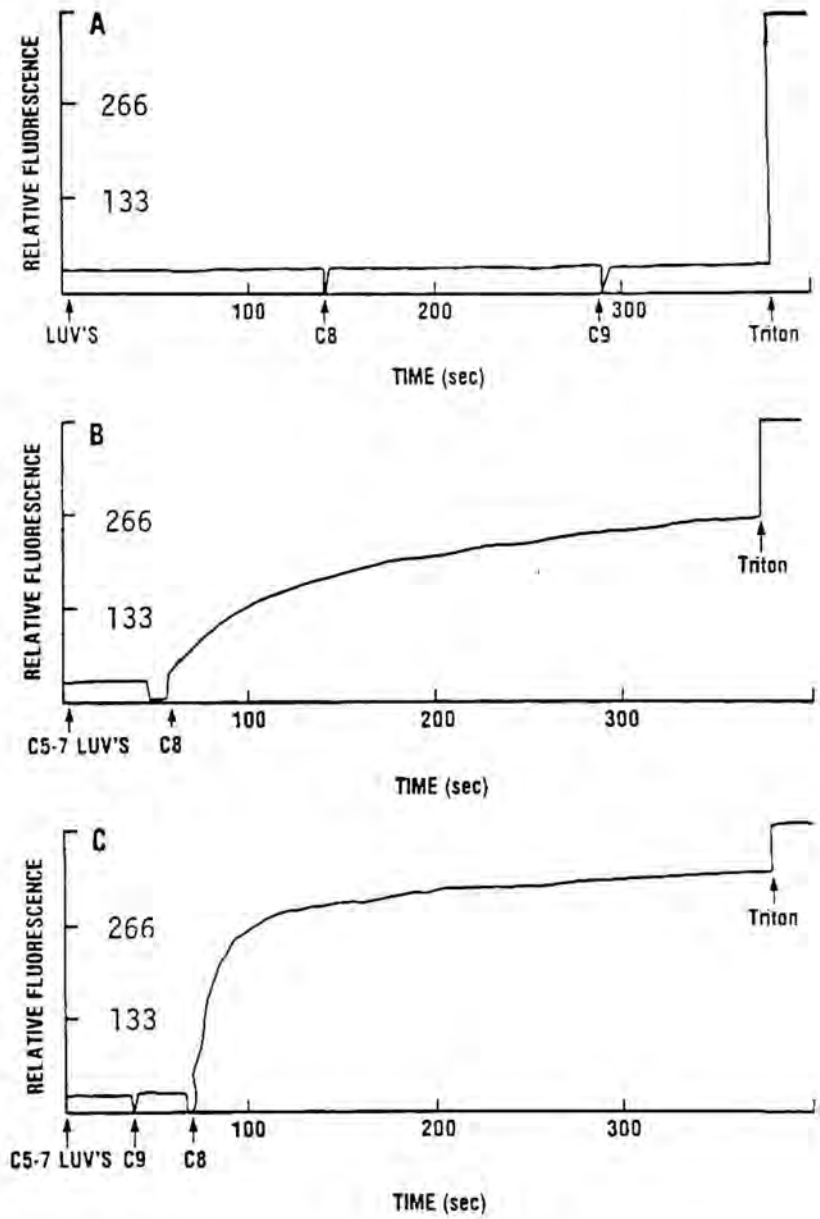

A
RELATIVE FLUORESCENCE
266
133
LUV'S
100
C8
200
C9
300
Triton
TIME (sec)
B
RELATIVE FLUORESCENCE
266
133
Triton
C5-7 LUV'S
C8
100
200
300
TIME (sec)
C
RELATIVE FLUORESCENCE
266
133
Triton
C5-7 LUV'S
C9
C8
100
200
300
TIME (sec)

Figure 15

Separation of Free C7 from C5-7 LUV's: C5-7 was assembled on 6CF-loaded LUV's as described in Materials and Methods. C5-7 treated LUV's (100 ug lipid) were run over a 6 ml Sepharose-4B column. Vesicles bearing C5-7 complexes were eluted by centrifugation of the column at 400 rpm for 15 seconds at 4 °C. Bound and free C7 was determined by ^{3}H-C7. Solid bars: fractions containing lipids. Open bars: fractions containing ^{3}H-C7. LUV lipid concentration was determined by 14C-dipalmitoyl-phosphatidylcholine.

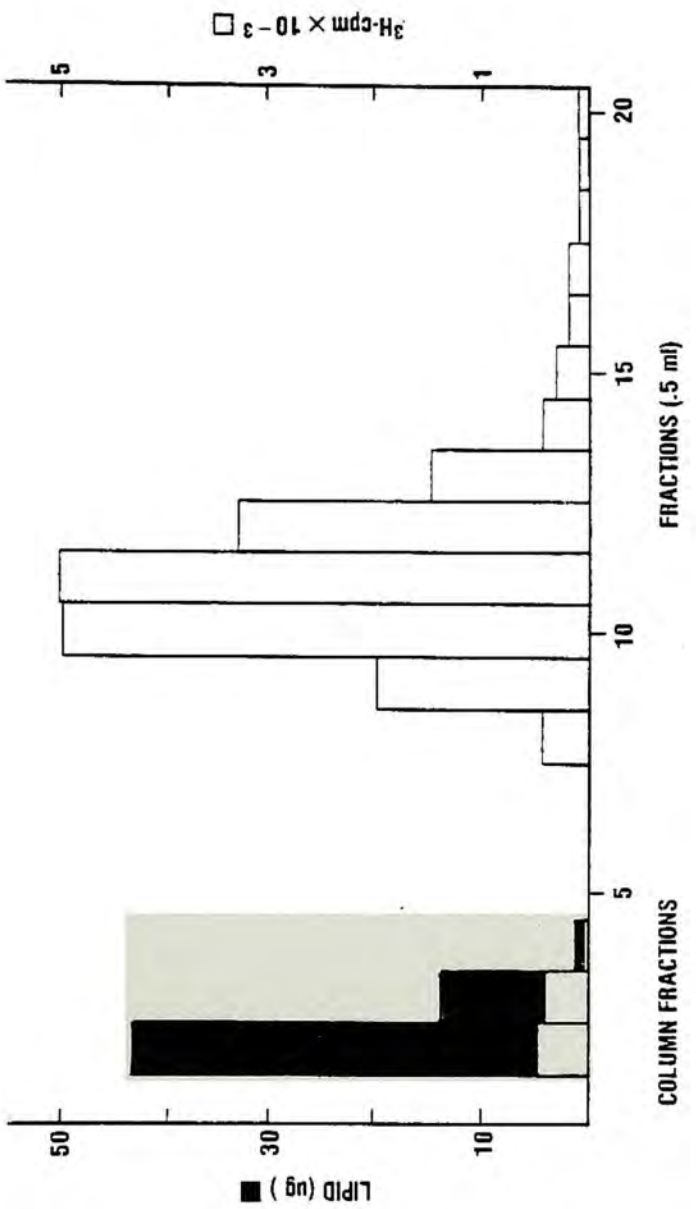
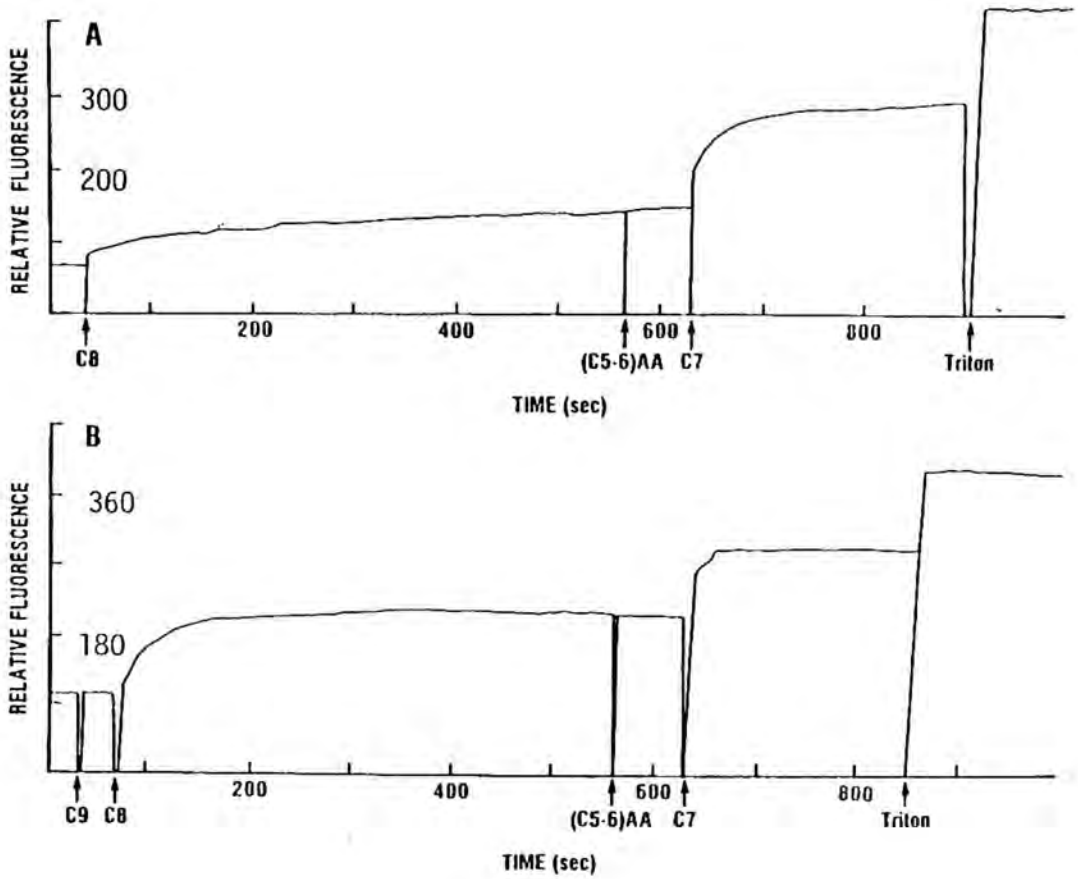

LIPID (ug) ■
50
30
10
COLUMN FRACTIONS
5
10
15
20
FRACTIONS (.5 ml)
3H-cpm × 10 − 3 □
5
3
1

Figure 16

Increased 6CF Release by the Assembly of Additional Complement Complexes on C5-8 and C5-9 Treated LUV's: C5-8 and C5-9 complexes were assembled on LUV's containing 100 mM 6CF as described in Materials and Methods. 6CF release from C5-8-treated (A) and C5-9-treated (B) LUV's was monitored at 37 °C. Additional complement complexes were assembled on vesicles by the addition (arrows) of acid-activated C5-6 (C5-6 AA) and C7 in the presence of excess C8 and C9.


A
RELATIVE FLUORESCENCE
300
200
200
400
600
800
C8
(C5-6)AA
C7
Triton
TIME (sec)
B
RELATIVE FLUORESCENCE
360
180
200
400
600
800
C9
C8
(C5-6)AA
C7
Triton
TIME (sec)

of more C5-8 or C5-9 complexes deposited on the membrane indicated that 6CF release was limited only by the number of pre-assembled C5-7 complexes and not by a peculiar resistance of some vesicles to complement attack.

Optimization of Conditions Required for C5-8 and C5-9 Assembly and Activity on LUV's

1. Acid-Activation

The first and most critical step in the assembly process was the acid-activation of C5-6. The following experiments were performed to determine if there was an optimum pH of acid activation. Multiple Samples of C5-6 were acid-activated at different pH values and the C5-9 complexes were assembled on LUV's. The percent of 6CF release clearly showed dependence on the pH of acid-activation (Table I) with an acid-activation at pH 5.5 resulting in optimal release. Photolabeling studies were performed to determine if the efficiency of insertion of C5-6 and or C7 into membranes was pH dependent. For these studies, C5-6 and C5-7 were assembled on LUV's containing the membrane-restricted photoprobe, 12-APS-GlcN. Interestingly, insertion of C5 and C6 into LUV's did not show a dependence on the pH of acid-activation

Table I. Percent release of 6CF from C5-9-Treated Vesicles as a Function of Acid-Activation of C56[a]

		pH of Acid-Activation							
		7.5	7.0	6.5	6.0	5.5	5.0	4.5	4.0
1.	% Release of 6CF[b]		12 ± 1	16 ± 1	N.D.	29 ± 1.2	N.D.		
2.	% Release of 6CF[b]		15	29	30	43	41		
3.	% Release 6CF	12.6 ± 1.4	N.D.	21 ± 0.8	N.D.	53 ± 5	N.D.	29.5 ± 0.5	0

a. The assembly of C5-9 complexes on LUV's (loaded with 100 mM 6CF) and the measurement of C5-9 functional activity were performed as described in Materials and Methods. Multiple samples of C5-9 were acid-activated at different pH values. 1, 2 and 5 represent results of three experiments.

b. Determined by following 6CF release to endpoint in a spectrofluorometer. 100% release was determined by the addition of 2% Triton X-100 to vesicles. Values represent the average of duplicate samples.

(Table II). However, the insertion of C7 did increase as the pH of C5-6 acid-activation was progressively decreased, with maximum C7 insertion occurring with acid-activation of C5-6 under pH 6 (Table II). Thus, rather than directly facilitating C5-6 insertion into membranes, acid-activation of C5-6 at lower pH values may expose the C7 binding site(s) on C5-6 more efficiently. Since the results indicated that acid-activation of C5-6 at pH 5.5 led to optimal C5-9 functional activity, all subsequent C5-6 acid-activations were done at pH 5.5.

It has been shown that complement could be more efficiently deposited on RBC membranes if C5-6 was acid activated in the presence of RBCs (74). Therefore it was necessary to determine if the presence of LUV's during the acid-activation step also enhanced assembly on vesicles. Samples of C5-6 were acid-activated either in the presence of LUV's or in the absence of LUV's with subsequent addition to vesicles. The remaining proteins were added directly to the vesicles as usual. As shown in Table III, C5-8 and C5-9 complexes assembled from C5-6 acid-activated in the absence of vesicles had greater functional activity than complexes assembled from C5-6 acid-activated in the presence of LUV's. Thus, all subsequent

Table II. Insertion of Complement Proteins as a Function of the pH of Acid-Activation[a]

	pH				
	7.0	6.5	6.0	5.5	5.0
Insertion Index of C5-6[b]	4.0	5.1	4.8	4.8	5.2
Ratio of Inserted C5:C6[c]	2.5	2.4	2.5	2.4	2.4
Insertion Index of C5-6[b]	4.4	4.0	4.2	4.0	4.2
Ratio of Inserted C5:C6[c]	2.7	2.3	2.3	2.1	2.4
Ratio of Inserted C7:C5-6[d]	0.12	0.13	0.21	0.28	.29

a. C5-6 was acid-activated as described in Materials and Methods. Multiple samples of C5-6 were acid-activated at different pH values. The photoreactive probe 12-APS-GlcN was incubated with LUV's for 15 minutes at 37 °C before the addition of complement proteins. Acid-activated C5-6 or C5-7 was added to membranes as described in Materials and Methods and the samples were irradiated after incubation. The membranes were pelleted at 108,000 x g for 30 minutes, run on 7.5% polyacrylamide slab gels and transferred to nitrocellulose. After staining with amido black, protein bands were cut out of the nitrocellulose paper and counted in a liquid scintillation counter to obtain the amount of radioactivity associated with each protein.

b. The insertion index of C5-6 was defined as the cpm in the C5-6 bands divided by the weight of paper corresponding to the area defined by the densitometric scan of the amido black-stained C6 band on the blot.

c. The insertion ratio C5:C6 was defined as: $\frac{\text{cpm in C5}}{\text{cpm in C6}}$

d. The insertion ratio C7:C5-6 was expressed as: $\frac{\text{cpm in C7}}{\text{cpm in C5 + C6}}$

Table III. C5-9 Complex Activity as a Function of the Acid-Activation of C5-6 in the Presence or Absence of LUV's

Acid-Activation of C5-6[a]		C5-9 Functional Activity	
		% 6CF Release[b]	Rate of 6CF Release[c]
1.	+ LUV's	36	539
2.	- LUV's	51	688

a. C5-6 was acid-activated to pH 5.5 as described in Materials and Methods either in the presence of LUV's (1) or alone, in the absence of LUV's (2).

b. Determined by following 6CF release to endpoint in a spectrofluorometer. 100% release determined by the addition of 2% Triton X-100 to vesicles.

c. Determined from the slope of the line drawn tangentially to the steepest portion of the dye release curve (as described in Materials and Methods) and expressed as $\Delta F/\Delta t$ (fluorescence units/second).

experiments were performed with acid-activation of C5-6 prior to incubation with vesicles.

2. The Efficiency of C5-7 Formation was Affected by the Order of Addition of Acid-activated C5-6, LUV's and C7.

Samples of acid-activated C5-6, LUV's and C7 were incubated either together at 37 °C for 15 minutes, or acid-activated C5-6 was incubated with vesicles for 10 minutes at 37 °C followed by a 5 minute, 37 °C incubation with C7. C5-8 and C5-9 complexes were then assembled on these two sets of C5-7 vesicles and functional activity was determined by the 6CF release assay. As shown in Table IV, higher activities were obtained by exposure of acid-activated C5-6 to vesicles before C7 addition. Moreover, the pH at which C7 was added to LUV's also affected C5-8 and C5-9 activity, with C5-7 assembly on vesicles at pH 7.4 resulting in maximum activity (Table V). In summary, the conditions required for optimal assembly and maximal activity of complement complexes on 6CF-loaded LUV's were: acid-activation of C5-6 to pH 5.5 followed by a 10 minute incubation at 37 °C of acid-activated C5-6 with LUV's, followed by an additional 5 minute, 37 °C incubation with C7 in PBS at pH 7.4.

Table IV. C5-8 and C5-9 Complex Activity as a Function of the Time of C7 Addition to C5-6 LUV's

Time of Addition of C7[a]	C5-8 Functional Activity		C5-9 Functional Activity	
	% 6CF Release[b]	Rate of 6CF Release[c]	% 6CF Release[b]	Rate of 6CF Release[c]
During the Incubation of Acid-Activated C5-6 with LUV's	22	23	39	225
After the Incubation of Acid-Activation of C5-6 with LUV's	31	56	45	281

a. C5-6 was acid-activated as described in Materials and Methods. C7 was incubated with acid-activated C5-6 and vesicles either for 15 minutes at 37 °C or for 5 minutes at 37 °C following a prior incubation of acid-activated C5-6 with LUV's for 10 minutes at 37 °C.

b. Determined by following 6CF release to endpoint in a spectroflurometer. 100% release determined by the addition of 2% Triton X-100 to vesicles.

C. Determined as described in Materials and Methods and expressed as $\Delta F/\Delta t$ (fluorescence units/second).

Table V. C5-9 Complex Activity as a Function of the pH of C5-7 Incubation

Incubation pH[a]	C5-9 Functional Activity	
	% 6CF Release[b]	Rate of 6CF Release[c] (Fluorescence Units/Second)
5.0	74	1160
5.5	69	1120
6.0	70	1340
7.0	83	1460
8.0	61	880

a. C5-7 was assembled on LUV's loaded with 100 mM 6CF as described in Materials and Methods. Multiple samples of C5-6 LUV's were incubated with C7 at different pH values.

b. Determined by following 6CF release to endpoint in a spectrofluorometer. 100% release determined by the addition of 2% Triton X-100 to vesicles.

c. Determined as described in Materials and Methods and expressed as $\Delta F/\Delta t$ (fluorescence units/second).

3. Estimation of the Number of C5-7 Complexes Bound per Vesicle

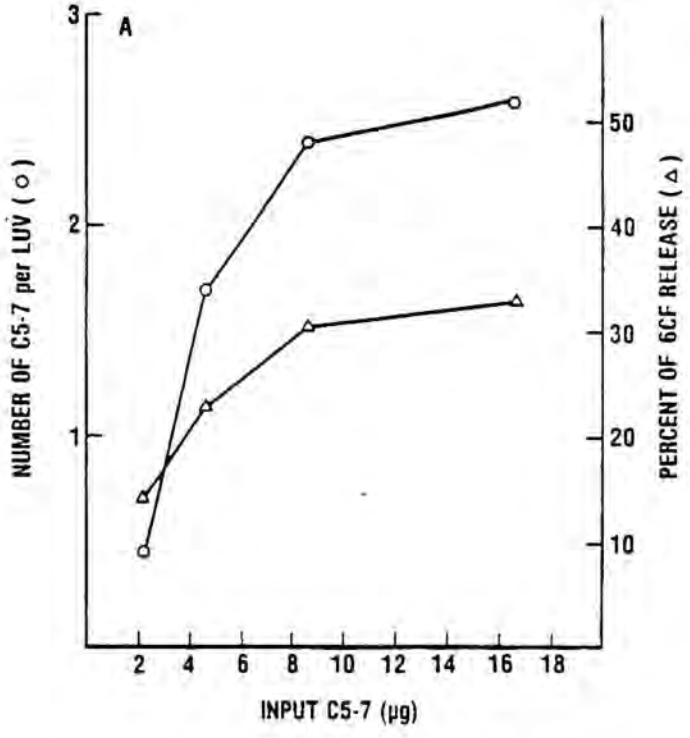
Under conditions of optimal C5-7 assembly on LUV's, the number of C5-7 complexes bound per vesicle as a function of C5, C6, and C7 input was determined. As shown in Figure 17, (for a given addition of C5-7), the amount of C5-7 bound to 6CF-loaded vesicles as well as the percent dye release from these vesicles with C5-9 lesions, increased with C5-7 input but reached a saturation level. The efficiency of protein binding to vesicles, however, was very low (5% of input C5-7) and represented an estimated 2 to 10 C5-7 complexes per LUV assuming an average vesicle diameter of 2500 Å. Nevertheless, the % 6CF release was directly proportional to the estimated number of complexes bound per vesicle (Fig. 17B).

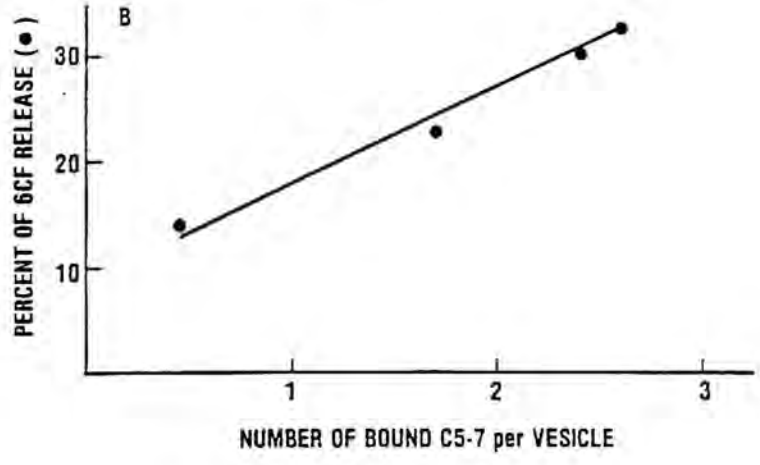
4. Determination of C5-7 LUV, C8 and C9 Concentrations Required for Optimal Functional Activity

The amount of C5-7 vesicle lipid, C8 and C9 required for maximal complement complex activity was determined by measuring the percent and rate of 6CF release from C5-8 and C5-9 vesicles while independently varying C5-7 LUV, C8 and C9 input within practical

Figure 17

Binding of C5-7 to LUV's: Estimation of the number of C5-7 complexes per vesicle was determined as described in Materials and Methods. (A) Number of C5-7 complexes per vesicle and percent 6CF release are plotted as a function of the amount of C5-7 added to LUV's. (B) Percent 6CF release is plotted as a function of the number of estimated C5-7 complexes per LUV.


A
NUMBER OF C5-7 per LUV (○)
PERCENT OF 6CF RELEASE (△)
INPUT C5-7 (μg)
3
2
1
50
40
30
20
10
2 4 6 8 10 12 14 16 18


B
PERCENT OF 6CF RELEASE (●)
NUMBER OF BOUND C5-7 per VESICLE
30
20
10
1
2
3

limits. The results of these independent titrations were that optimal functional activity was achieved when 3 ug of C8, (C5-8 complex) or 3 ug C8 and 3 ug C9 (C5-9 complex) were added to 10 ug of C5-7 LUV lipid (Tables VI and VII).

Lifetime of C5-7 Complexes on Vesicles

The lifetime of C5-7 complexes on vesicles was determined by measuring the percent and rate of 6CF release from C5-8 and C5-9 lesions as a function of elapsed time after assembly of C5-7 complexes on vesicles. The percent and rate of dye release remained constant up to 48 hours after C5-7 assembly on LUV's when assayed for C5-9 functional activity (Table VII). In contrast, the rate of dye release from C5-8 lesions 48 hours post-C5-7 assembly was 85% of the rate obtained with newly assembled C5-7 complexes even though the percent 6CF release did not change. These data indicate that C5-7 complexes are relatively stable on membranes for at least 48 hours.

Path of 6CF Efflux from LUV's

Bramhall had previously shown that vesicles treated with gramicidin released 6CF (75). He hypothesized that 6CF diffused directly through the

Table VI. Determination of the Amount of C5-7 LUV Lipid Required for Optimal C5-8 and C5-9 Activity

C5-7 LUV LIPID[a]	C5-8 Functional Activity		C5-9 Functional Activity	
	% 6CF Release[b]	Rate of 6CF Release[c]	% 6CF Release[b]	Rate of 6CF Release[c]
2.5	31	25	37	125
5	34	94	40	218
10	38	156	51	688

a. Micrograms of C5-7 LUV lipid used in the functional activity assay as described in Materials and Methods, 3 ugs of C8 and C9 (molar excess) were added to C5-7 LUV's.

b. Determined by following 6CF release to endpoint in a spectrofluorometer. 100% release determined by the addition of 2% Triton X-100 to vesicles.

C. Determined as described in Materials and Methods and expressed as $\Delta F/\Delta t$ (fluorescence units/second).

Table VII. Determination of the Amount of C8 and C9 Required to Saturate C5-7 complexes deposited on LUV's

Micrograms C8	Micrograms C9	C5-9 Functional Activity: % 6CF Release[a]	C5-9 Functional Activity: Rate of 6CF Release[b]
1	1	.34	250
2	2	35	375
3	3	36	539
4	4	36	469

a. Determined by following 6CF release to endpoint in a spectrofluorometer. 100% release determined by the addition of 2% Triton X-100 to vesicles.

b. Determined as described in Materials and Methods and expressed as $\Delta F/\Delta t$ (fluorescence units/second).

Table VIII. Lifetime of C5-7 Complexes on LUV's

Time (hours) Post C5-7 Assembly[a]	C5-8 Functional Activity		C5-9 Functional Activity	
	% Release[b]	Rate of 6CF Release[c]	% Release[b]	Rate of 6CF Release[c]
0	18 ± 0.5	80 ± 2.5	36 ± 0.5	231 ± 6.5
24	16 ± 0.85	69 ± 2.5	34 ± 0.5	257 ± 32
48	16 ± 0.40	62 ± 2.9	34 ± 1	219 ± 19

a. Functional activity of C5-8 and C5-9 complexes was measured as a function of the elapsed time (hours) after C5-7 assembly on LUV's. Assembly of C5-7 on vesicles and functional assay were performed as described in Materials and Methods. Values represent the average of duplicate samples.

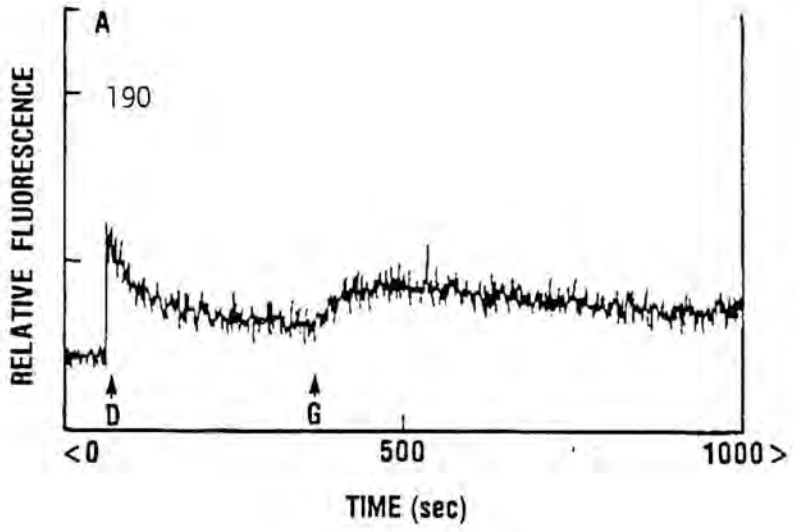
b. Determined by following 6CF release to endpoint in a spectrofluorometer. 100% release determined by the addition of 2% Triton X-100 to vesicles.

c. Determined as described in Materials and Methods and expressed as $\Delta F/\Delta t$ (fluorescence units/second).

membrane bilayer since 6CF is too large to penetrate through the gramicidin channel. He argued that free diffusion of 6CF through the bilayer in the absence of gramicidin was inhibited by the rapid buildup of a membrane diffusion potential due to the negative charge on 6CF. Thus, gramicidin facilitated 6CF diffusion by allowing the co-migration of positive counter ions, thereby preventing any buildup of a membrane potential. To further test this hypothesis, the interaction of gramicidin with 6CF-loaded LUV's prepared by reverse phase evaporation was investigated. Ion channels induced by gramicidin were detected by the change in fluorescence of the membrane potential-sensitive probe, diS-C_3-(5) (68). The magnitude of the fluorescence change depended on the ion gradient between the extra- and intravesicular media as well as on gramicidin concentration. The maximum fluorescence change of diS-C_3-(5) was achieved under conditions described in Figure 18. Concentrations of gramicidin which induced ion channels did not release 6CF (Figure 18). Higher concentrations of gramicidin that did release 6CF also released the same percentage of entrapped sucrose (Table IX). These results indicated that 6CF release was most likely mediated through a generalized membrane perturbation caused by a possible detergent-like effect

Figure 18

LUV's Treated With Gramicidin: (A) Ion channels induced by gramicidin were detected by the change in diS-C_3-(5) fluorescence. 1 ug of diS-C_3-(5) was added to 300 ug LUV lipid (containing 100 mM 6CF (Na salt)) in 2 ml of K-PBS (150 mM KCl, 5 mM K_2HPO_4). Maximum membrane potential (depolarization) was induced by the addition of 2 ug of gramicidin. Relative fluorescence (excitation 622 nm, emission 670 nm) was measured as a function of time. "D" indicates time of addition of dye and G, the time of addition of gramicidin. (B) Addition of gramicidin to LUV's containing 100 mM 6CF, 0.1 ug diS-C_3-(5) and 0.2 ug gramicidin were added to 30 ug LUV lipid in 2 ml K-PBS. Relative fluorescence (excitation 490 nm, emission 520) was measured as a function of time. At the same gramicidin to lipid ratio as in (A), no 6CF was released after gramidicin addition.


A
190
RELATIVE FLUORESCENCE
D
G
<0
500
1000>
TIME (sec)

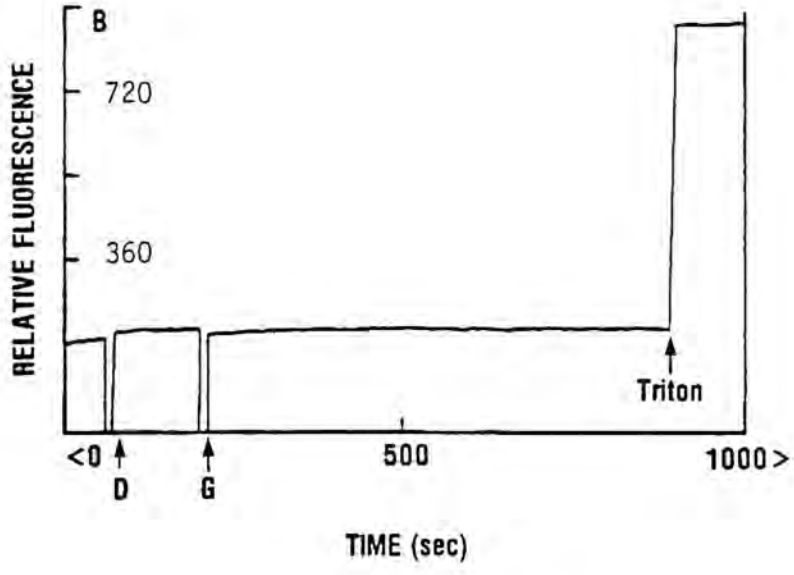

B
720
360
RELATIVE FLUORESCENCE
Triton
<0
D
G
500
1000>
TIME (sec)

Table IX. Release of 6CF and Sucrose from LUV's Treated with Gramicidin

	Gramicidin[a](ug)	% 6CF Release[b]	% Sucrose Release[c]
1.	0.01	0	0
	10	15	17
	20	34	28
2.	0.034	0.7	0
	0.17	5	5
	3	11	11
	21	40	32

a. Data show results from two experiments in which Gramicidin was added to 30 ug LUV loaded with 100 mM 6CF and sucrose (1) or added to 10 ug LUV loaded with 30 mM 6CF and sucrose (2).

b. Determined by following 6CF release to endpoint on a spectrofluorometer. 100% release determined by the addition of 2% Triton X-100 to vesicles.

c. Determined by comparing the residual ^{3}H-sucrose associated with vesicle fractions to that of an untreated LUV control following the removal of released ^{3}H-sucrose with a Sepharose-4B column.

of gramicidin on vesicle membranes and not by gramicidin-induced collapse of the membrane potential. If this interpretation is correct, then the data implies that the release of 6CF from complement-treated LUV's may occur either through a perturbation in the membrane caused by complement complexes, or directly through the protein complexes themselves.

II. Properties of the C5-8 Complex: Comparison with C5-9 and C5-9_1 Complexes

Dose Response of C5-8 and C5-9 Functional Activity to Complement Concentration

It was of interest to determine if the LUV model system would respond to complement in the same manner as natural membranes. Therefore, the functional activities of both C5-8 and C5-9 complexes assembled on LUV's was measured as a function of complement concentration. When the amount of C5-7 added to vesicles was titrated, the percent and rate of 6CF release from C5-8-treated vesicles was dependent on the input of C5-7 (Figures 19 and 20). Under conditions in which vesicles were given a single treatment of C5-7, there was no further increase in the rate of 6CF release and the % release appeared to level off above 20 ug of added C5-7. This is probably attributable to

Figure 19

Dose Response of C5-8-Induced 6CF Release to C5-7 Concentration: The percent of 6CF release (% lysis) by C5-8 was measured as a function of the amount of C5-7 assembled on vesicles. C5-7 complexes were assembled on 6CF-loaded LUV's at different concentrations of C5, C6 and C7. The molar ratio of C5, C6 and C7 was 1:1:1 in all cases. The amount of C5-7 input noted in the figure represents the sum of the amounts of these 3 proteins incubated with 100 ug vesicle lipid. After passage of each sample through 6 ml Sepharose-4B minicolumns, C8-induced release of 6CF was initiated by the addition of the indicated amount of C8 directly to 10 ug of vesicle lipid (bearing C5-7 complexes) in 2 ml of buffer in a spectrofluorometer cuvette.

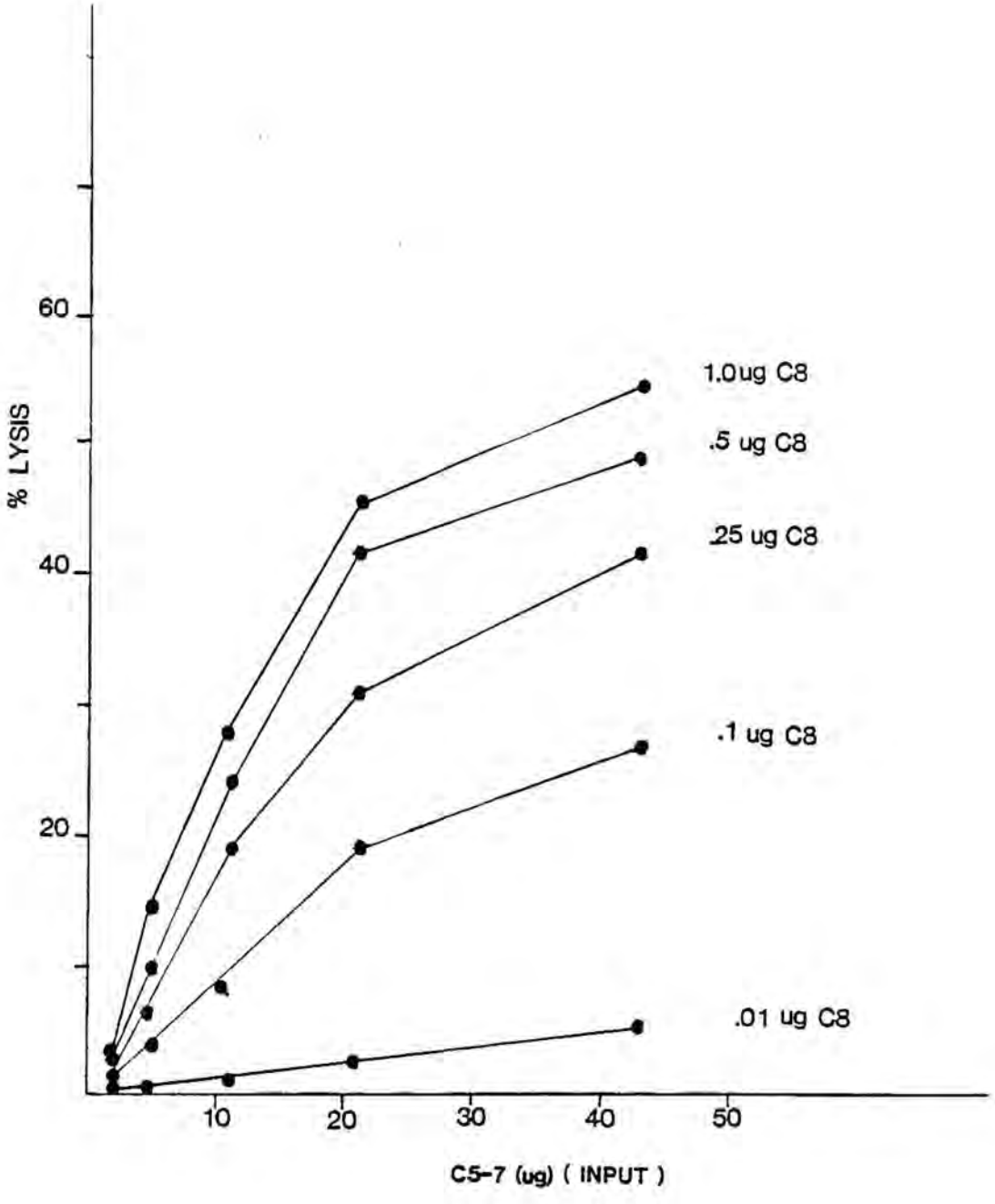
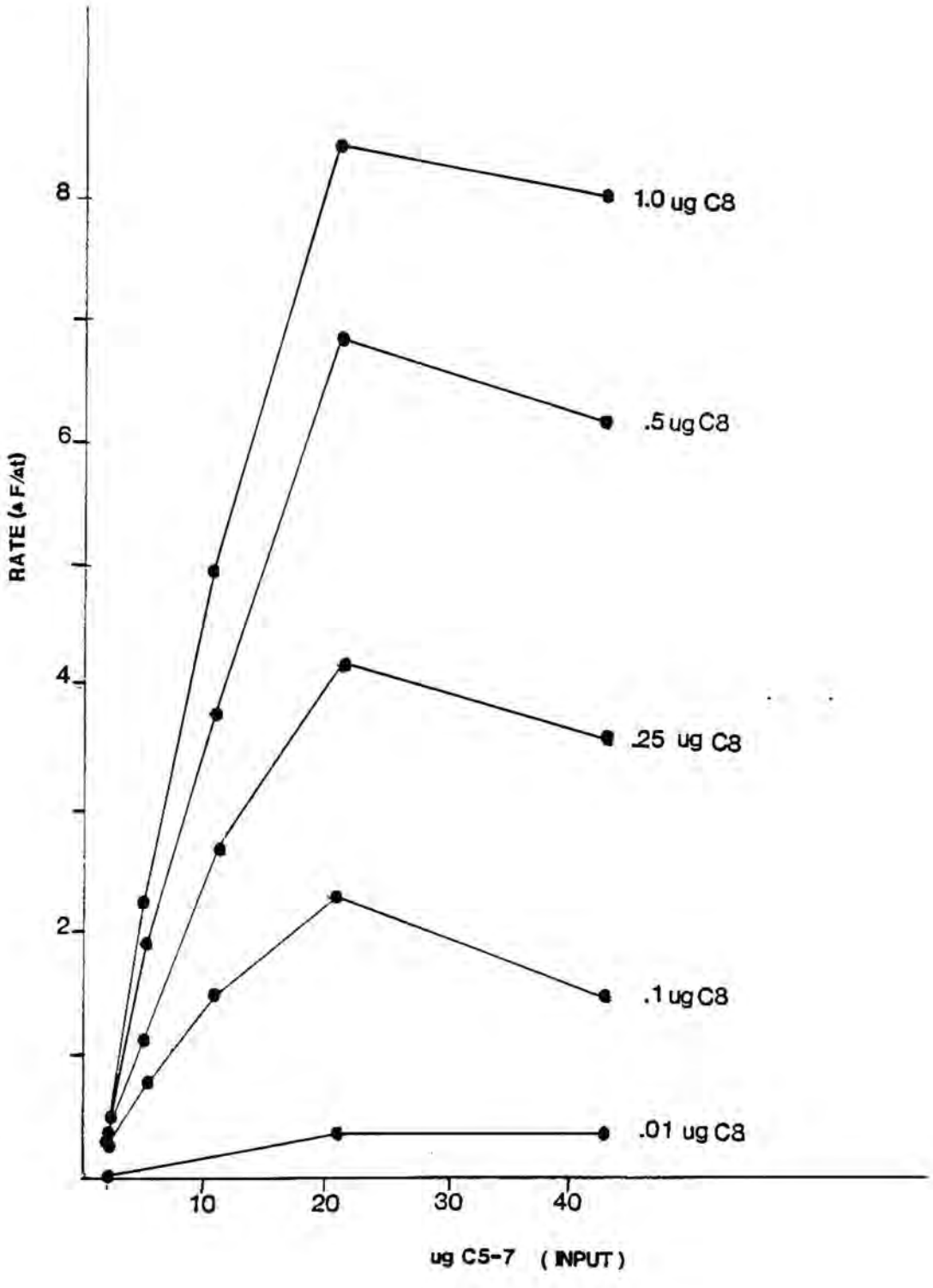

% LYSIS
60
40
20
10
20
30
40
50
C5-7 (ug) (INPUT)
1.0ug C8
.5 ug C8
.25 ug C8
.1 ug C8
.01 ug C8

Figure 20

Dose Response of the rate of C5-8-Induced 6CF Release to C5-7 Concentration: The rate of 6CF release by C5-8 was measured as a function of the amount of C5-7 assembled on vesicles. Details of the C5-7 assembly process are provided in the legend of Figure 18.


RATE (Δ F/Δt)
8
6
4
2
10
20
30
40
ug C5-7 (INPUT)
1.0 ug C8
.5 ug C8
.25 ug C8
.1 ug C8
.01 ug C8

the saturation in C5-7 binding. Similarly, when C8 was titrated, both the percent and rate of 6CF release was dependent on C8 concentration (Figures 21 and 22). Functional activity began to level off at C8 input greater than 0.5 ug. Like that of the C5-8 complex, the functional activity of the C5-9 complex on LUV's was dependent on C5-7 input (Figures 23 and 24). Table X shows the dose response of C5-9-induced 6CF release to C8 and C9 concentrations. These data were used to determine the concentrations of C8 and C9 which represented a functional excess relative to the amount of C5-7 vesicles used.

It was of interest to determine the duration of 6CF release from complement-treated LUV's. More specifically we investigated whether dye release from C5-8 or C5-9-treated vesicles reached a finite endpoint at which dye efflux ceased. 6CF release from C5-8 and C5-9-treated LUV's reached an endpoint usually 1200 seconds and 500 seconds, respectively after the addition of C8, or C8 or C9 to C5-7 LUV's. As shown in Figure 25, after endpoint dye release was reached for both C5-8- and C5-9-treated LUV's, no further 6CF was released from these vesicles during a period of at least 30 minutes.

As shown in Table XI, C9 enhanced the percent and rate of 6CF release induced by the C5-8 complex.

Figure 21

Dose Response of C5-8-induced 6CF release to added C8: The percent of 6CF release (% lysis) by C5-8 was measured as a function of C8 input. C5-7 complexes were assembled on 6CF-loaded LUV's as described in the legend to Figure 18. The % of 6CF release was determined for varying amounts of C8 added to C5-7 vesicle samples.

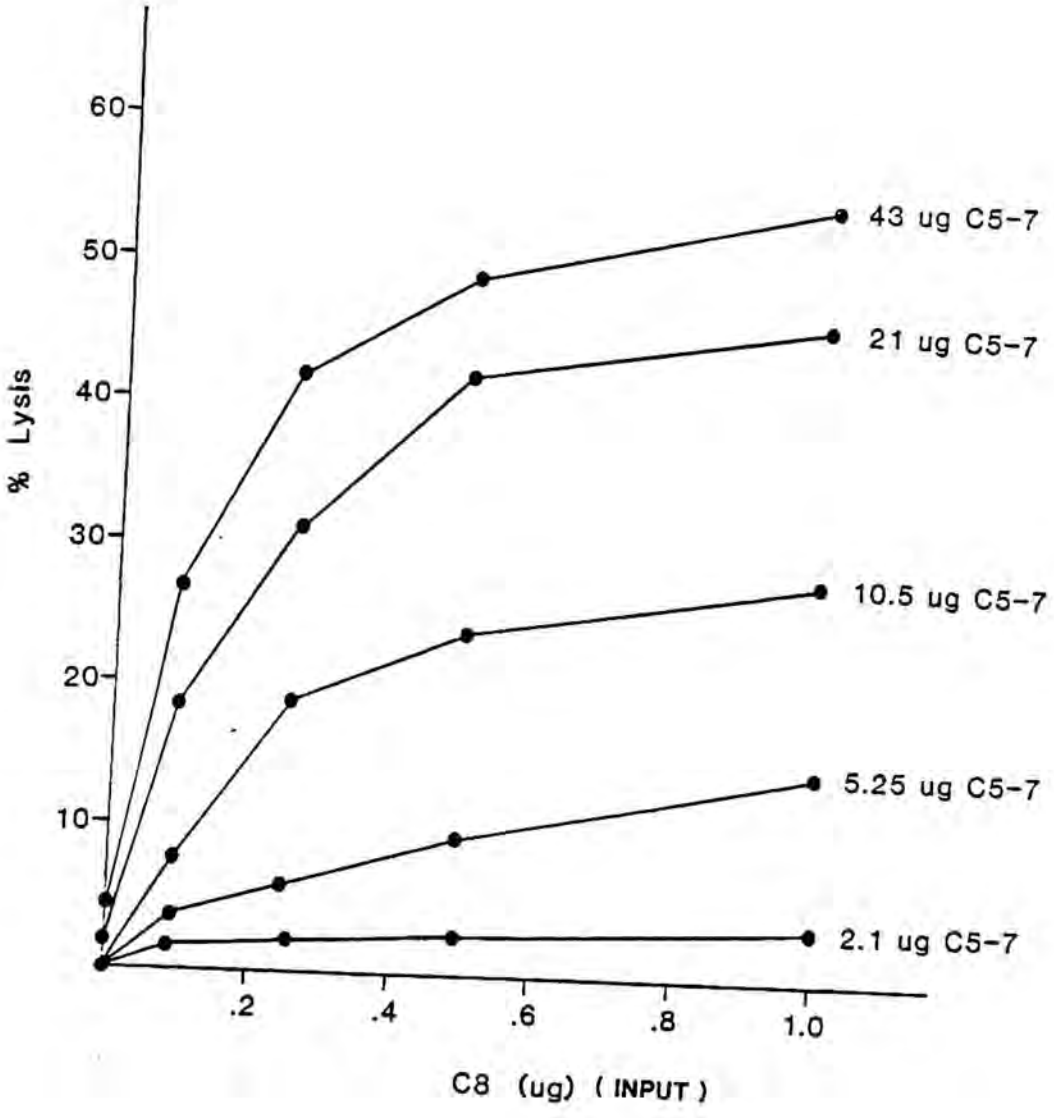

60
50
40
30
20
10
% Lysis
43 ug C5-7
21 ug C5-7
10.5 ug C5-7
5.25 ug C5-7
2.1 ug C5-7
.2
.4
.6
.8
1.0
C8 (ug) (INPUT)

Figure 22

Dose Response of the rate of C5-8-induced 6CF release to added C8: The rate of 6CF release by C5-8 was measured as a function of C8 input. C5-7 complexes were assembled on 6CF-loaded LUV's as described in the legend to Figures 19 and 21. The rate of C5-8-induced release was determined upon the addition of varying amounts of C8 to the C5-7 vesicle samples.

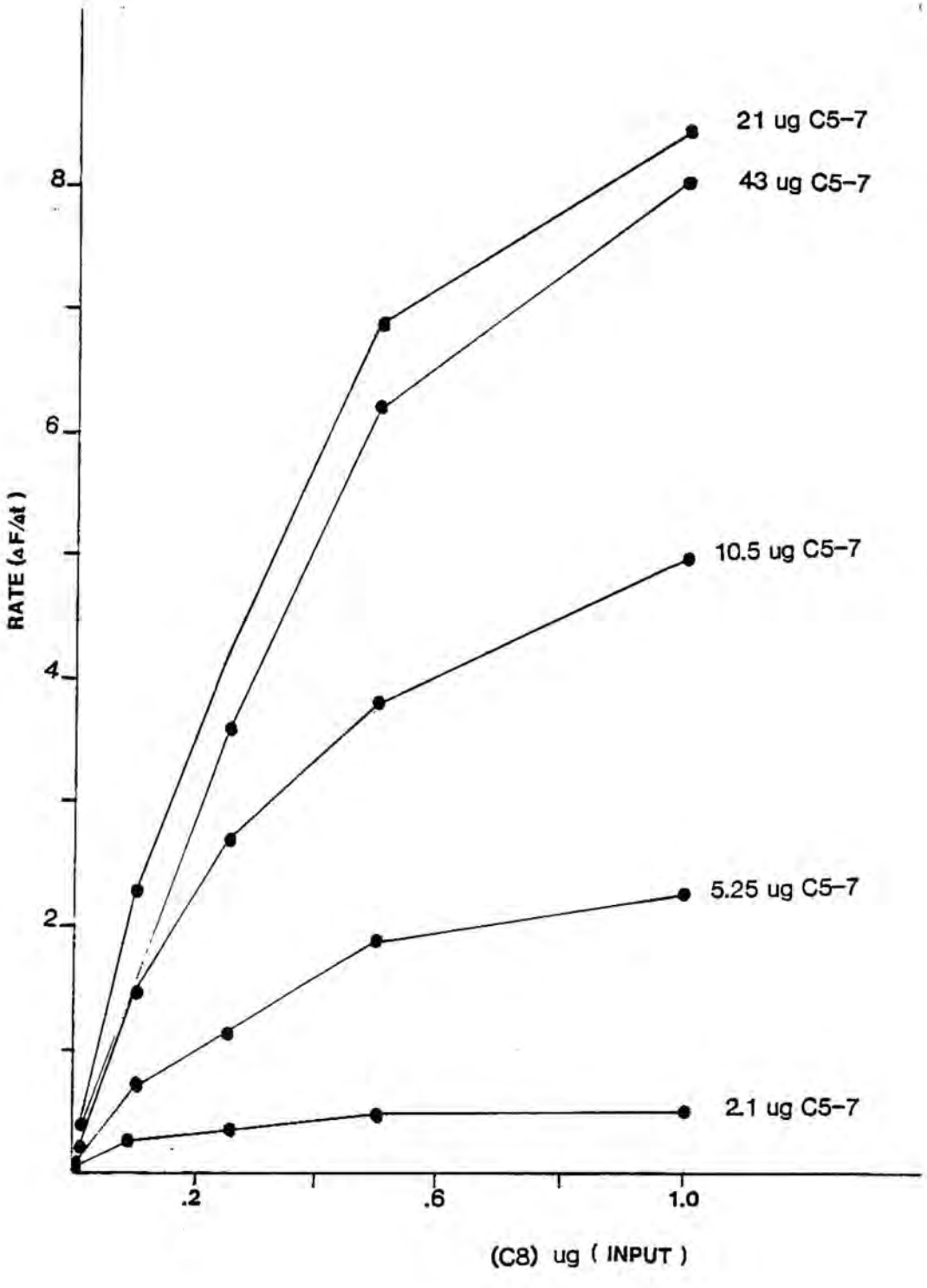

21 ug C5-7
43 ug C5-7
10.5 ug C5-7
5.25 ug C5-7
2.1 ug C5-7
RATE (ΔF/Δt)
8
6
4
2
.2
.6
1.0
(C8) ug (INPUT)

Figure 23

Dose Response of the Percent of C5-9-Induced 6CF Release to C5-7 concentration: The percent of 6CF release of C5-9 treated vesicles was measured as a function of C5-7 input. C5-7 complexes were assembled on 6CF-loaded LUV's as described in Materials and Methods. Excess C8 and C9 were added to 10 ug LUV lipid samples bearing different amounts of input C5-7.

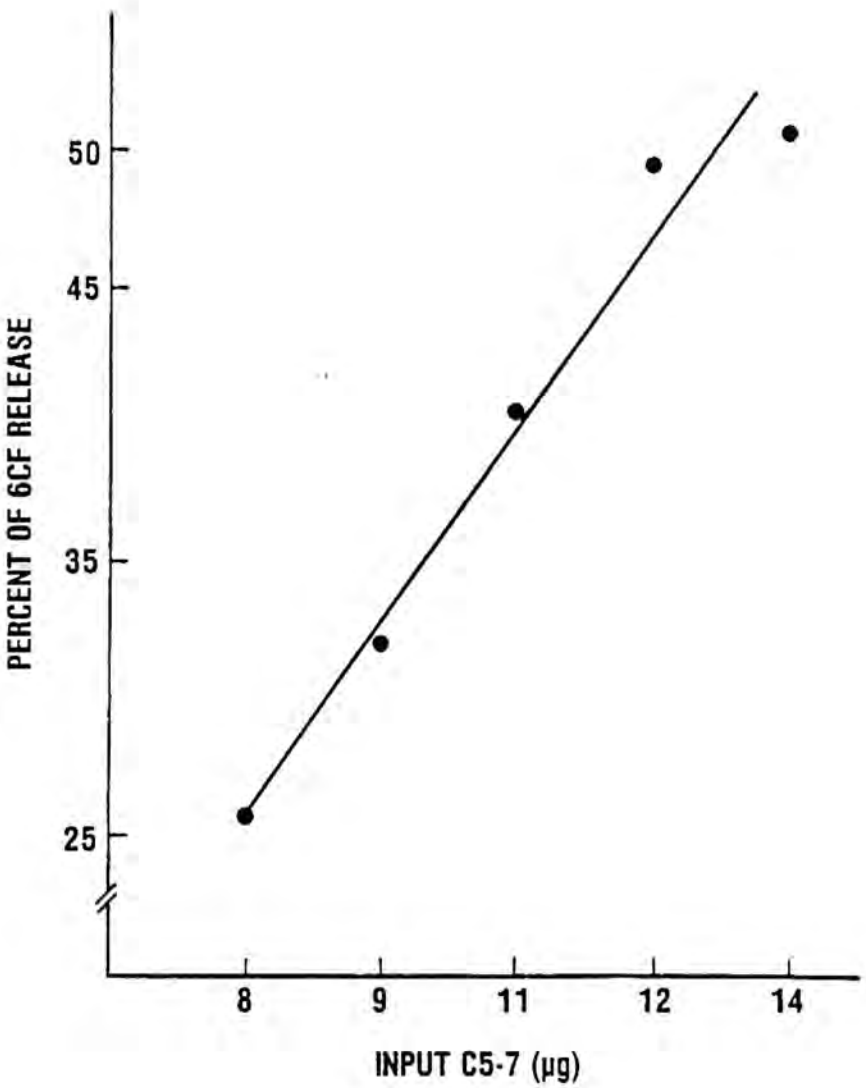

PERCENT OF 6CF RELEASE
50
45
35
25
8
9
11
12
14
INPUT C5-7 (µg)

Figure 24

Dose Response of the Rate of C5-9-induced 6CF Release to C5-7 Concentration: The rate of 6CF release from C5-9-treated vesicles was measured as a function of C5-7 input. C5-9 complexes were assembled on 6CF-loaded LUV's as described in the legend to Figure 23.

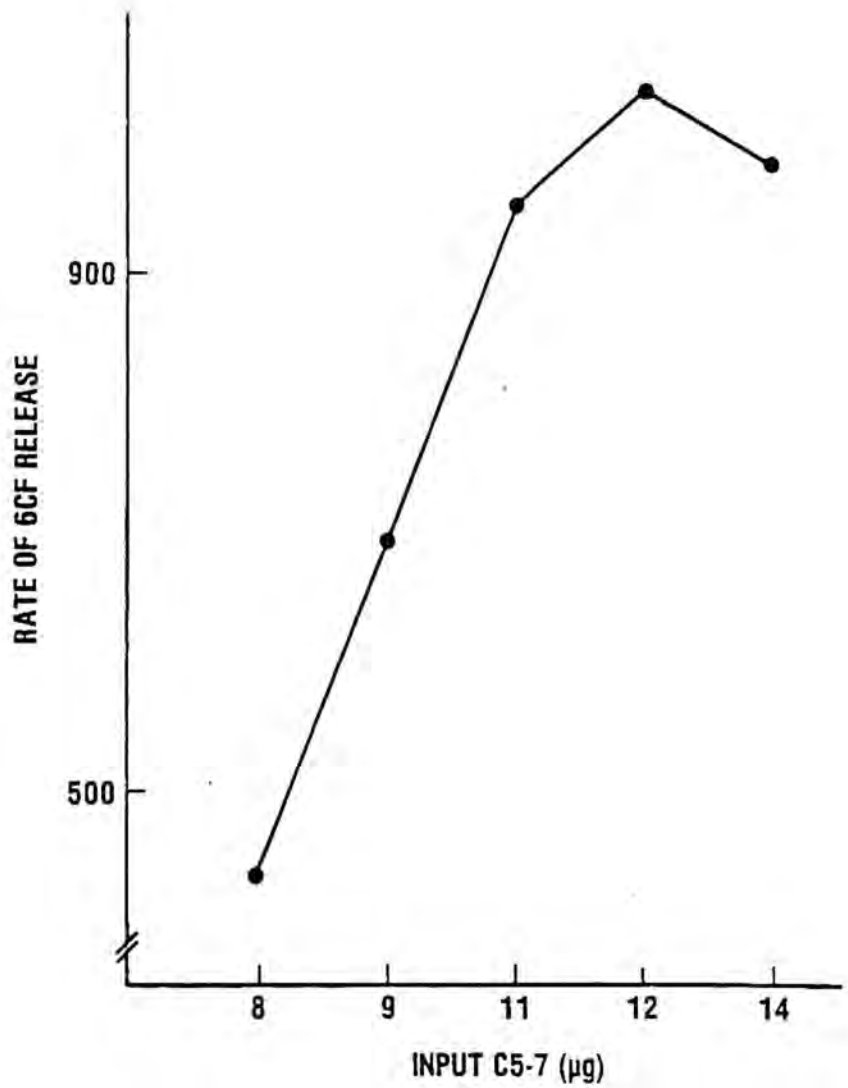

RATE OF 6CF RELEASE
900
500
8
9
11
12
14
INPUT C5-7 (µg)

Table X. Dose Response of C5-9 Functional Activity to Input C8 and C9

Titrated Component[a]	ug	C5-9 Functional Activity (6CF Release)[b]
1 C8	3	32
	2	32
	1	32
	0.5	32
	0.25	30
	0.05	25
	0.01	8
2 C9	3	31
	1	31
	0.5	32
	0.1	29
	0.025	31
	0.01	20

a. C5-7 was assembled on LUV's as described in Materials and Methods. The dose response of C5-9 complex activity was measured as a function of C8 input (1) under conditions in which 4 and 2 ug of C5-7 LUV lipid and C9 were used respectively, and C9 input (2) in which 4 and 1 ug of C5-7 LUV lipid and C8 were used respectively.

b. Determined by following 6CF release to endpoint in a spectrofluorometer. 100% release determined by the addition of 2% Triton X-100 to vesicles.

Figure 25

Determination of Endpoint 6CF Release From C5-8- and C5-9-treated LUV's: LUV's contained 30 mM 6CF. C5-8 and C5-9 complexes were assembled on 6CF loaded LUV's as described in Materials and Methods. The endpoint of dye release was determined as the time (following the addition of C8 or C8 and C9 to C5-7 LUV's) when the efflux of 6CF from these vesicles ceased.

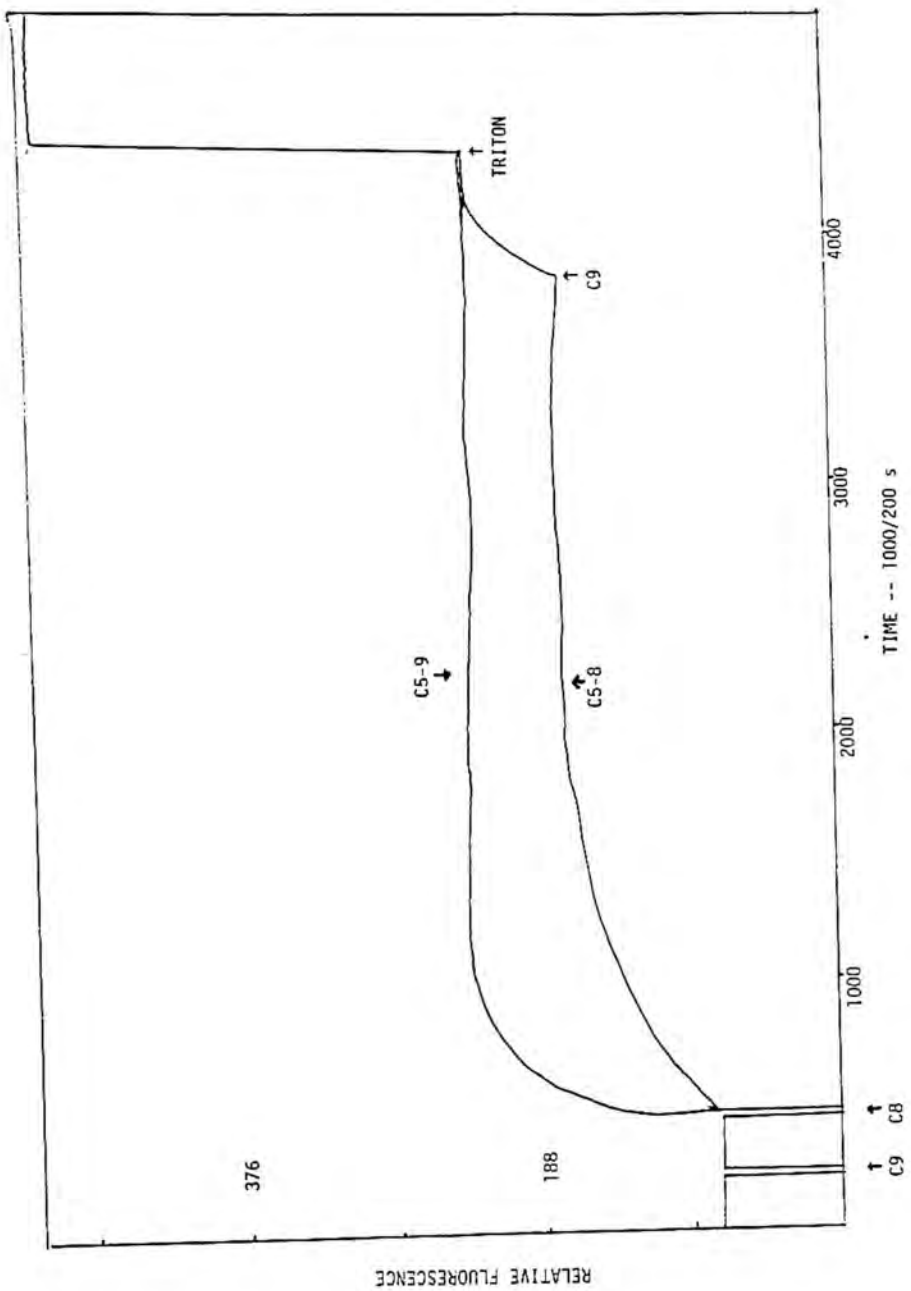

TRITON
C9
C5-9
C5-8
C9
C8
376
188
1000
2000
3000
4000
TIME -- 1000/200 s
RELATIVE FLUORESCENCE

Table XI. Comparison of C5-8 and C5-9 Functional Activity

Input C5-7[a] (ug C5-7)	C5-8 Functional Activity		C5-9 Functional Activity	
	% 6CF Release[b]	Rate of 6CF Release[c]	% 6CF Release[b]	Rate of 6CF Release[c]
8	8	19	19	413
9	15	40	30	563
11	21	57	36	656
12	28	112	42	693
14	36	177	48	750

a. C5-8 and C5-9 complexes were assembled on LUV's as described in Materials and Methods. Percent and rate of 6CF release from C5-8 and C5-9 LUV's were measured as a function of input C5-7 added to LUV's. 10 ug of C5-7 LUV lipid were used in these assays.

b. Determined by following 6CF release to endpoint in a spectrofluorometer. 100% release determined by the addition of 2% Triton X-100 to vesicles.

c. Determined as described in Materials and Methods and expressed as $\Delta F/\Delta t$ (fluorescence units/second).

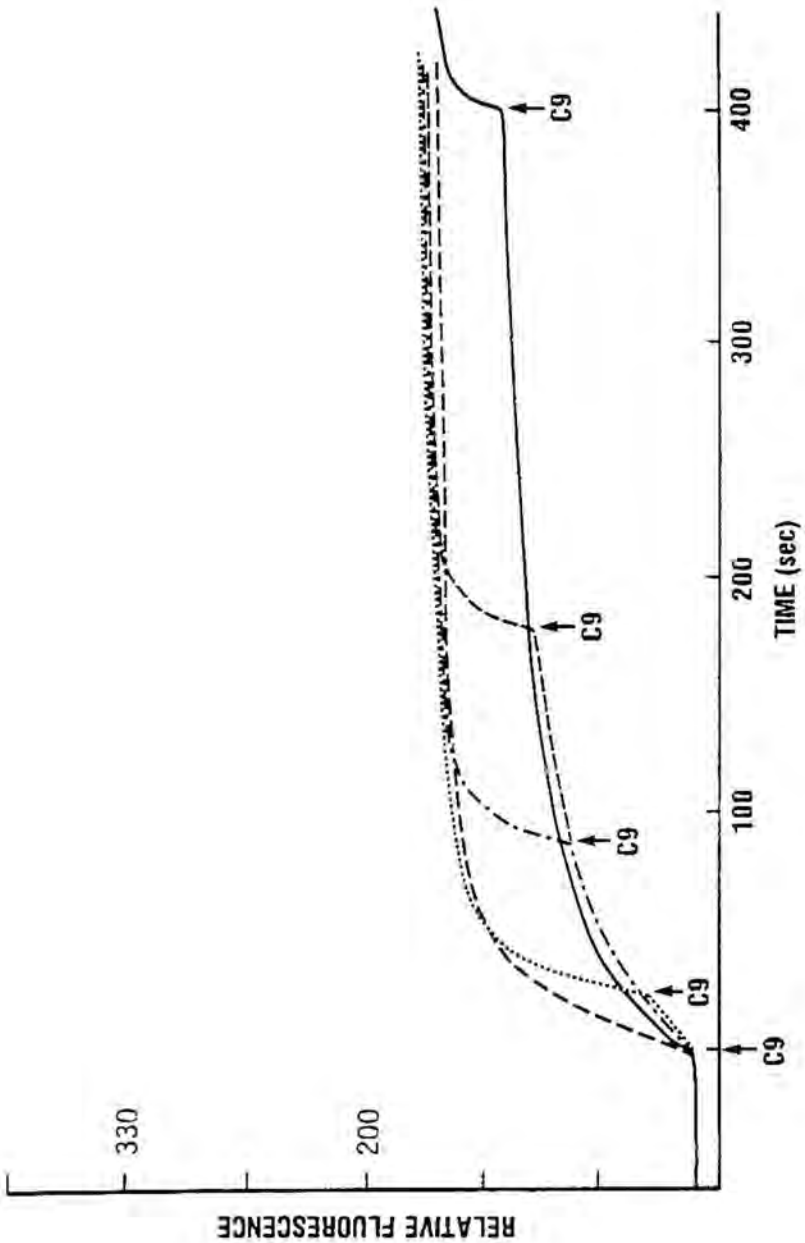
Moreover, C5-8 complexes possessed stable C9 binding sites, since the time of C9 addition following the initiation of 6CF release by C5-8 complexes, did not affect endpoint dye release (Figures 25 and 26). It is possible that the slower rate of release from C5-8-treated vesicles compared to that of C5-9-treated LUV's may result from differences in pore sizes of C5-8 and C5-9 complexes or LUV's. Nevertheless, the fact that less 6CF is released through C5-8 complexes than through C5-9 complexes suggests that the C5-8 channels close before total release of dye from a vesicle can be achieved. This interpretation is supported by studies on the mode of dye release (see below).

Determination of the Pore Size of C5-8 and C5-9 Complexes on Vesicles

It was of interest to determine the dimensions of the lesions created by C5-8 and C5-9 complexes. The pore sizes of the membrane-bound complexes were determined by molecular sieving studies using LUV's containing 6CF as well as tritiated sucrose (Stokes radius = 4.4 Å) and inulin (Stokes radius = 14.8 Å). C5-8 and C5-9 complexes were assembled on these vesicles and the release of these entrapped markers was measured. Sucrose, but

Figure 26

Addition of C9 at Different Times After the Initiation of C5-8-Mediated 6CF Release: C5-8 complexes were assembled on 6CF-loaded LUV's as described in Materials and Methods. 3 ug of C9 were added at different times (arrows) after the initiation of C5-8-induced 6CF release. 6CF release was monitored to endpoint at 37 °C.


RELATIVE FLUORESCENCE
330
200
TIME (sec)
100
200
300
400
C9
C9
C9
C9
C9

(Tables XII and XIII). This not inulin, was released from C5-8-treated LUV's indicated that the C5-8 lesion was at least 8.8 Å but not greater than 30 Å in diameter. Moreover, the percent of sucrose released was equivalent to the percent of released 6CF (Table XII), suggesting a common pathway for release. In contrast to C5-8 LUV's, vesicles treated with C5-9 released both sucrose and inulin which indicated that the size of the C5-9 complex was at least 30 Å in diameter. Although sucrose and 6CF were released to the same extent, inulin release from C5-9 vesicles was typically 50-75% of 6CF release. This disproportionate release of inulin and 6CF may indicate size heterogeneity of C5-9 lesion. As previously suggested (52, 53), the pore size may be related to the number of C9 molecules bound per C5-8 complex. These results on the relative pore size of the C5-8 and C5-9 complexes on vesicles compare well with results obtained from molecular sieving studies in which the release of entrapped sucrose or inulin was measured from complement-treated ghosts. C5-8-treated ghosts released sucrose, but not inulin, while C5-9-treated ghosts released both (32). From this, one can conclude that the structure of the C5-8 and C5-9 lesions on vesicles are similar to that formed on a natural target.

Table XII. Release of Encapsulated ^{3}H-Sucrose by LUV's Treated with Complement

Expt.	C5-8[a] % Release		C5-9[a] % Release	
	Sucrose[b]	6CF[c]	Sucrose[b]	6CF[c]
1	62	58	67	70
2	39	44	51	53
3	57	52	66	60

a. C5-8 and C5-9 complexes were assembled on LUV's loaded with 100 mM 6CF and radiolabeled sucrose as described in Materials and Methods. Three separate experiments are represented.

b. Determined by comparing the residual ^{3}H-sucrose associated with vesicles to that of the C5-7 control following the removal of released ^{3}H-sucrose with a Sepharose-4B column.

c. Determined by following 6CF release to endpoint on a spectrofluorometer. 100% release was determined by the addition of 2% Triton X-100 to the vesicles.

Table XIII. Release of Encapsulated ^{3}H-Inulin by LUV's Treated with Complement

Expt.	C5-8[a] % Release		C5-9[a] % Release	
	Inulin[b]	6CF[c]	Inulin[b]	6CF[c]
1	0	54	54	80
2	0	50	47	63
3	0	40	52	75

a. C5-8 and C5-9 complexes were assembled on LUV's loaded with 100 mM 6CF and radiolabeled inulin as described in Materials and Methods. Three separate experiments are represented.

b. Determined by comparing the residual ^{3}H-inulin associated with vesicles to that of the C5-7 control following the removal of released ^{3}H-inulin with a Sepharose-4B column.

c. Determined by following 6CF release to endpoint on a spectrofluorometer. 100% release was determined by the addition of 2% Triton X-100 to vesicles.

Determination of the Mode of Dye Release from LUV's

In characterizing dye release induced by C5-8 and C5-9 complexes, it was of interest to determine whether C5-8- and C5-9-treated vesicles released dye in a graded or all-or-none manner. C5-7, C5-8 and C5-9 complexes were assembled on 6CF-loaded LUV's as described in Materials and Methods. After monitoring dye release on a fluorometer, vesicles were separated from released 6CF by passage over a 6 ml Sepharose-4B column. Vesicle fractions were then analysed for residual 6CF in the absence and presence of Triton X-100 (i.e., the Fo/Ft ratio was determined). Table XIV shows representative experiments in which Fo/Ft ratios were determined (and compared to C5-7 controls) for C5-8 and C5-9 complexes assembled on LUV's containing three different concentrations of entrapped dye. In columns 3 and 4 are the Fo/Ft ratios expected for all-or-none and graded release, respectively. The measured Fo/Ft ratios of C5-8-treated vesicles showed values compatible with a graded release mechanism, while C5-9-treated LUV's had Fo/Ft ratios predicted for an all-or-none release. The graded mode of dye release induced by C5-8 was demonstrated more clearly under conditions of high C5-7 input in which 6CF release in the presence of C8 was greater than 40% (Table XV). Furthermore,.experiments in which the mode of dye

Table XIV. Internal Fluorescence After 6CF Release from LUV's Induced by C5-8 and C5-9

	Sample[a]	% 6CF Release[b]	Predicted Fo/Ft for all-or-none[c] release	Predicted Fo/Ft for Graded Release[d]	Measured Fo/Ft[e]
1	C5-7	0	-	-	0.088
	C5-8	40	0.088	0.14	0.186
	C5-9	45	0.088	0.145	0.08
2	C5-7	0	-	-	0.26
	C5-8	20	0.26	0.36	0.32
	C5-9	40	0.26	0.52	0.26
3	C5-7	0	-	-	0.49
	C5-8	27	0.49	0.64	0.65
	C5-9	42	0.49	0.72	0.48

a. Complexes assembled on LUV's loaded with 100 mM 6CF (1), 30 mM 6CF (2) and 20 mM 6CF (3) as described in Materials and Methods.

b. Determined by following 6CF release to endpoint on a spectrofluorometer. 100% release of 6CF was determined by the addition of 2% Triton X-100.

c. Assumes some vesicles released all their dye; others released none.

d. Predicted on the basis of a quenching curve determined by the method of Blumenthal et al. (65).

e. Determined following the removal of released 6CF with a Sepharose-4B column.

Table XV. Internal Fluorescence After 6CF Release from LUV's Induced by C5-8

	Sample[a]	% 6CF Release[b]	Predicted Fo/Ft for all-or-none[c] Release	Predicted Fo/Ft for Graded Release	Measured Fo/Ft
1	C5-7	0	-	-	0.13 ± 0.01
	C5-8	48	0.13	0.32	0.32 ± 0.01
2	C5-7	0	-	-	0.54 ± 0.005
	C5-8	43	0.54	0.73	0.74 ± 0.01
3	C5-7	0	-	-	0.54 ± 0.01
	C5-8	60	0.54	0.80	0.80 ± 0.01

a. Complexes assembled on LUV's loaded with (50 mM 6CF (1), and 20 mM 6CF (2) and (3) as described in Materials and Methods. Each sample represents the average of duplicates.

b. Determined by following 6CF release to endpoint on a spectrofluorometer. 100% release of 6CF was determined by the addition of 2% Triton X-100.

c. Assumes some vesicles released all their dye; others released none.

d. Predicted on the basis of a quenching curve determined by the method of Blumenthal et al. (65).

e. Determined following the removal of released 6CF with a Sepharose-4B Column.

release was determined 60 minutes following the endpoint of 6CF release confirmed that C5-8-treated vesicles still retained a fraction of the entrapped dye. Thus, the graded release of 6CF from C5-8-treated LUV's may be indicative of a transient, unstable perturbation or pore. The lesion induced by C5-8 is transient in the sense that once formed, it remains open for a period of approximately twenty minutes and then closes permanently.

Interestingly, under conditions of high C5-7 input in which 6CF release in the presence of C8 and C9 was greater than 50%, dye was released from LUV's in a graded fashion (data not shown). This anomalous result for C5-9-mediated marker release may be due to the possibility that at high C5-7 input, there may not be enough C9 to bind to all of the functional C5-8 complexes giving rise to a mixture of vesicles bearing either C5-8 or C5-9 complexes, thus leading to the apparently graded response. As will be shown below for the "C5-9_1" complex, the temperature at which C9 is assembled on the C5-8 complex also affects the mode of release.

In similar studies, the mode of dye release was also assessed for gramicidin-treated LUV's. As shown in Table XVI, the Fo/Ft ratios determined at different gramicidin concentrations were indicative of graded

Table XVI. Internal Fluorescence After 6CF Release from LUV's Induced by Gramicidin

Microgram Gramicidin Added[a] (ug)	% 6CF Release[b]	Predicted Fo/Ft for all-or-none[c] release	Predicted Fo/Ft for Graded Release[d]	Measured Fo/Ft[e]
0	0	---	---	0.108
0.034	0.7	0.108	0.11	0.11
0.17	5	0.108	0.11	0.115
3.0	11	0.108	0.12	0.129
21.0	40	0.108	0.19	0.197

a. Gramicidin added to 30 ug LUV's loaded with 100 mM 6CF.

b. Determined by following 6CF release to endpoint on a spectrofluorometer. 100% release of 6CF was determined by the addition of 2% Triton X-100.

c. Assumes some vesicles released all their dye; others released none.

d. Predicted on the basis of a quenching curve determined by the method of Blumenthal et al. (65).

e. Determined following the removal of released 6CF with a Sepharose-4B column.

release. Since the release of 6CF from gramicidin treated vesicles is most probably the result of a detergent-like effect, the graded release of 6CF from C5-8-treated vesicles may also be indicative of limited deterent action. At this point, it is not possible to distinguish this possibility from that of a transiently-open structural C5-8 channel.

Analysis of Complement-Treated LUV's by the Meridian ACAS 470 Fluorescence Work Station

Since a typical preparation of LUV's exhibits considerable size heterogeneity, it was of interest to determine whether the size of an LUV affects its interaction with the terminal complement proteins and hence its susceptibility to membrane attack. Conventional methods of vesicle separation, such as gel filtration chromatography, are not capable of resolving vesicles greater than 1000 Å and thus we developed a novel method of determining the size distribution of 6CF-loaded LUV's. Using this method, we were able to assess the lytic susceptibility of vesicles of different sizes as well as to confirm the observation reported in the previous section that the C5-8 complex induces a graded release of dye from vesicles.

The Meridian ACAS 470 Fluorescence Workstation was used to quantitate the fluorescence of 6CF within

individual LUV's. The rationale for the use of the Meridian system was that, since the vesicle fluorescence should be proportional to the volume of entrapped dye, the distribution of particle fluorescence within a vesicle population should be a reflection of the size heterogeneity of that population. Therefore, even though vesicles of ≈ 2500 Å are below the resolution of the light microscope, preparations of LUV's loaded with 100 mM 6CF were still visible on the CRT of the ACAS 470 Workstation as a distribution of individual particles with fluorescence intensities proportional to the entrapped volume or vesicle size (Figure 27). In comparing the histogram of vesicle distribution as a function of fluorescence intensity (Figure 28) to the histogram of vesicle distribution as a function of size as determined by EM (Figure 8), particles in the fluorescence intensity range of 16 to 30 (39% of the population) roughly correspond to vesicles in the 2000 to 3000 Å size range which represents about 40% of the population. Futhermore, vesicles in the size range 1000 to 4000 Å (79% of total) roughly correspond to vesicles with fluorescence intensities from 0 to 45 (80% of total population). As shown in Figure 29, the ACAS system could also distinguish large-sized LUV's from vesicle aggregates, induced by treating the

Figure 27

A Population of Carboxyfluorescein-loaded LUV's as Seen on the CRT Screen of the Meridian ACAS 470 Fluorescence Workstation: The vesicles were examined on glass slides which were coated with 0.025% poly-L-lysine which served to anchor the vesicles as well as keep them in the same focal plane. Each vesicle is represented by a single colored pixel. The color is a measure of fluorescence intensity. The entire field covers an area of 272,160 square microns. Each pixel corresponds to approximately 3 microns. The instrumental parameters were: PMT - 44%; X-step - 3 microns; Y-step - 3 microns; X points - 180; Y points- 168; scan strength - 6%; stage speed - 2 mm/sec; laser power - 200 mW. A 90% cutoff neutral density filter was used for all experiments. Scanning the field at smaller step sizes (eg., 0.25-1 micron) showed no evidence that any particle was greater than 1 micron in diameter.

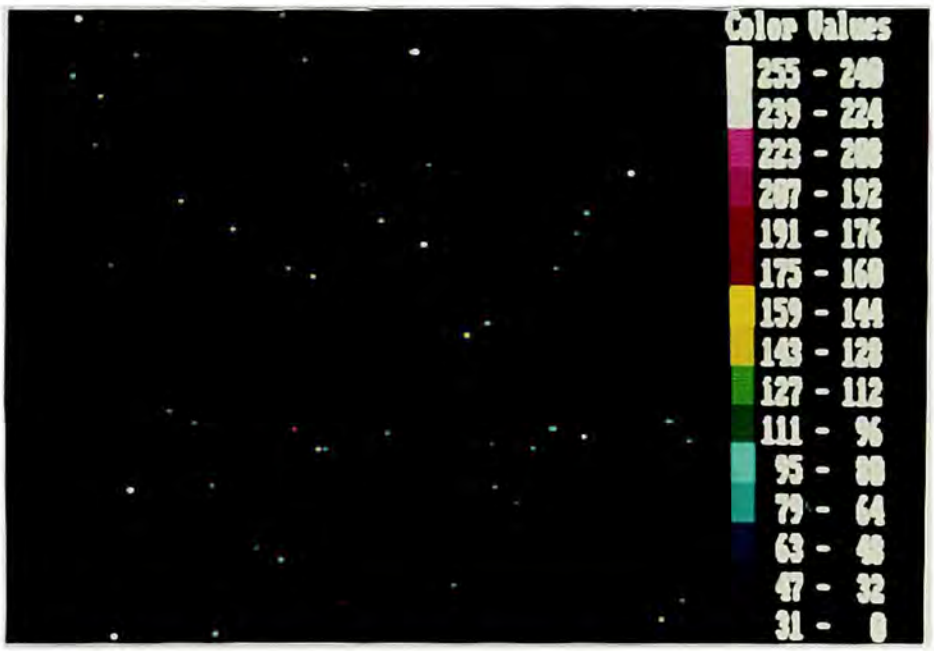

Color Values
255 - 240
239 - 224
223 - 208
207 - 192
191 - 176
175 - 160
159 - 144
143 - 128
127 - 112
111 - 96
95 - 80
79 - 64
63 - 48
47 - 32
31 - 0

Figure 28

Fluorescence Profile for a Population of LUV's: The percentage of total particles is plotted as a function of fluorescence intensity. Each division on the x-axis represents 15 fluorescence units. These data were obtained using the FLINT and HISTOGRAM programs of the ACAS 470. Instrumental parameters were as stated in the legend of Figure 25. An average of 600 particles were analyzed per sample.

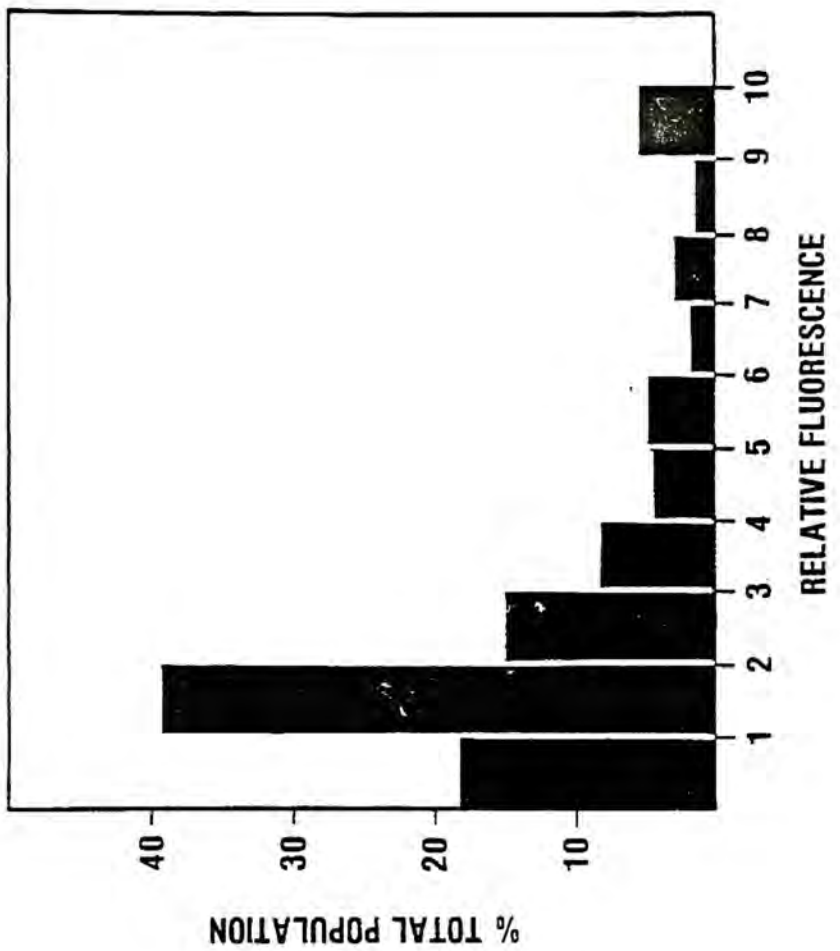
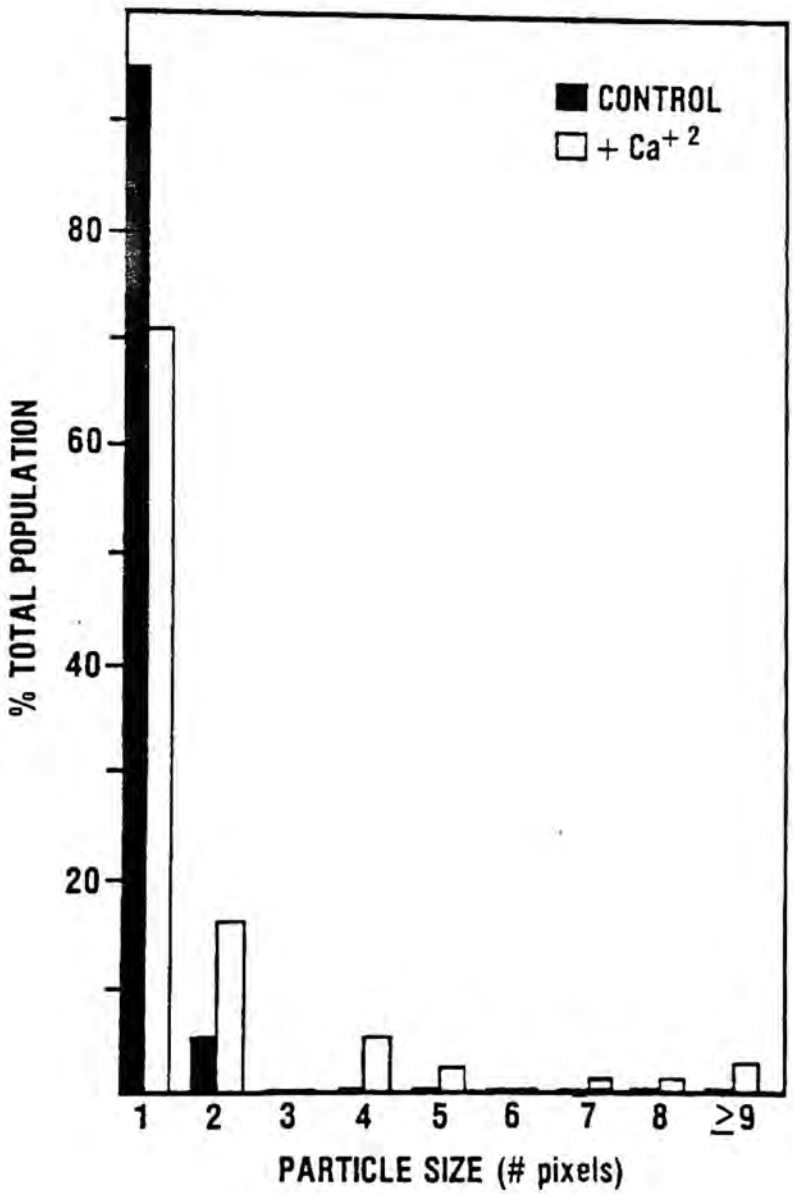

% TOTAL POPULATION
40
30
20
10
1
2
3
4
5
6
7
8
9
10
RELATIVE FLUORESCENCE

Figure 29

<u>Ca^{+2}-Induced Aggregation of Vesicles</u>: $CaCl_2$ (1 mM) was added to 10 ug lipid of LUV's to induce aggregation of these negatively charged vesicles. The histogram shows the increase in particle size upon addition of Ca^{+2} to the vesicle suspension. Solid bars: control; open bars: with Ca^{+2}

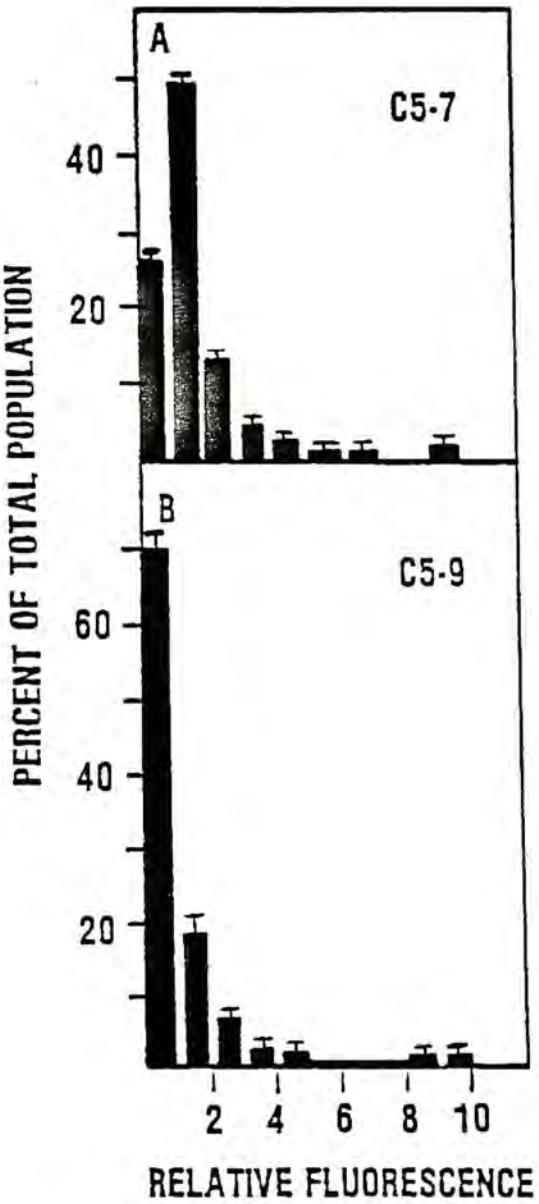

CONTROL
+ Ca+ 2
% TOTAL POPULATION
80
60
40
20
1
2
3
4
5
6
7
8
≥9
PARTICLE SIZE (# pixels)

negatively-charged LUV's with Ca^{++}. Thus, the correlation between vesicle fluorescence and vesicle size as well as the ability of the instrument to differentiate individual LUV's and vesicle aggregates permitted the use of the ACAS 470 Workstation as a tool for analyzing vesicle size distribution on the basis of entrapped fluorescence.

To assess the effect of vesicle size on the lytic susceptibility to complement, the fluorescence distribution of LUV's was determined before and after lysis by C5-9. C5-9 complexes were assembled on LUV's containing 100 mM 6CF and the release of dye was monitored to endpoint on a spectrofluorometer. After removal of free dye, aliquots of C5-9 and control (C5-7) LUV's were analysed for fluorescence distribution using the Meridian system (Figure 30). Lysis by the C5-9 complex resulted in an overall decrease in the number of fluorescent vesicles even though the lipid concentration of the C5-9 samples was equivalent to that of the C5-7 sample. This decrease in the number of vesicles containing entrapped dye was accompanied by a shift in the distribution of the remaining dye-containing vesicles towards the lowest intensity or smallest particles, suggesting that preferential lysis of the medium- and larger-sized

Figure 30

Fluorescence profiles of LUV's with C5-7 complexes and C5-9 complexes. The PTM setting for this set of experiments was increased to 51% for greater sensitivity. Each division on the x-axis represents 25 fluorescence units.


A
C5-7
40
20
B
C5-9
60
40
20
PERCENT OF TOTAL POPULATION
2
4
6
8
10
RELATIVE FLUORESCENCE

vesicles may occur to some extent, but that the majority of vesicles are susceptible to lysis by C5-9.

In addition to determining the lytic susceptibility of vesicles of different sizes, the ACAS system could be used to indicate dye release by a graded mechanism. Graded release of dye from vesicles with entrapped dye originally at a self-quenching concentration would lead to dilution of the remaining lumenal dye and therefore to a predicted increase in the fluorescence intensity of individual vesicles due to dequenching. This prediction was tested by analyzing vesicles with dye entrapped at 100 mM and 30 mM and was verified by the results shown in Figure 31. When analyzed at the same instrument settings, the fluorescence of vesicles containing 30 mM 6CF is greater than the fluorescence intensities of vesicles containing 100 mM 6CF, despite the fact that the size heterogeneity of the two vesicle preparations was the same. Therefore, a decrease in entrapped 6CF concentration (from self-quenching values) as a result of a graded release would be expected to cause an upward shift in the distribution of the vesicle population towards higher intensities. Figure 32 shows the fluorescence intensity profiles of vesicles treated with C5-7 and C5-8. As shown, C5-8-mediated lysis leads to a shift in the fluorescence profile towards a

Figure 31

Fluorescence profiles of (a) LUV's containing 30 mM CF, (b) LUV's containing 100 mM CF.

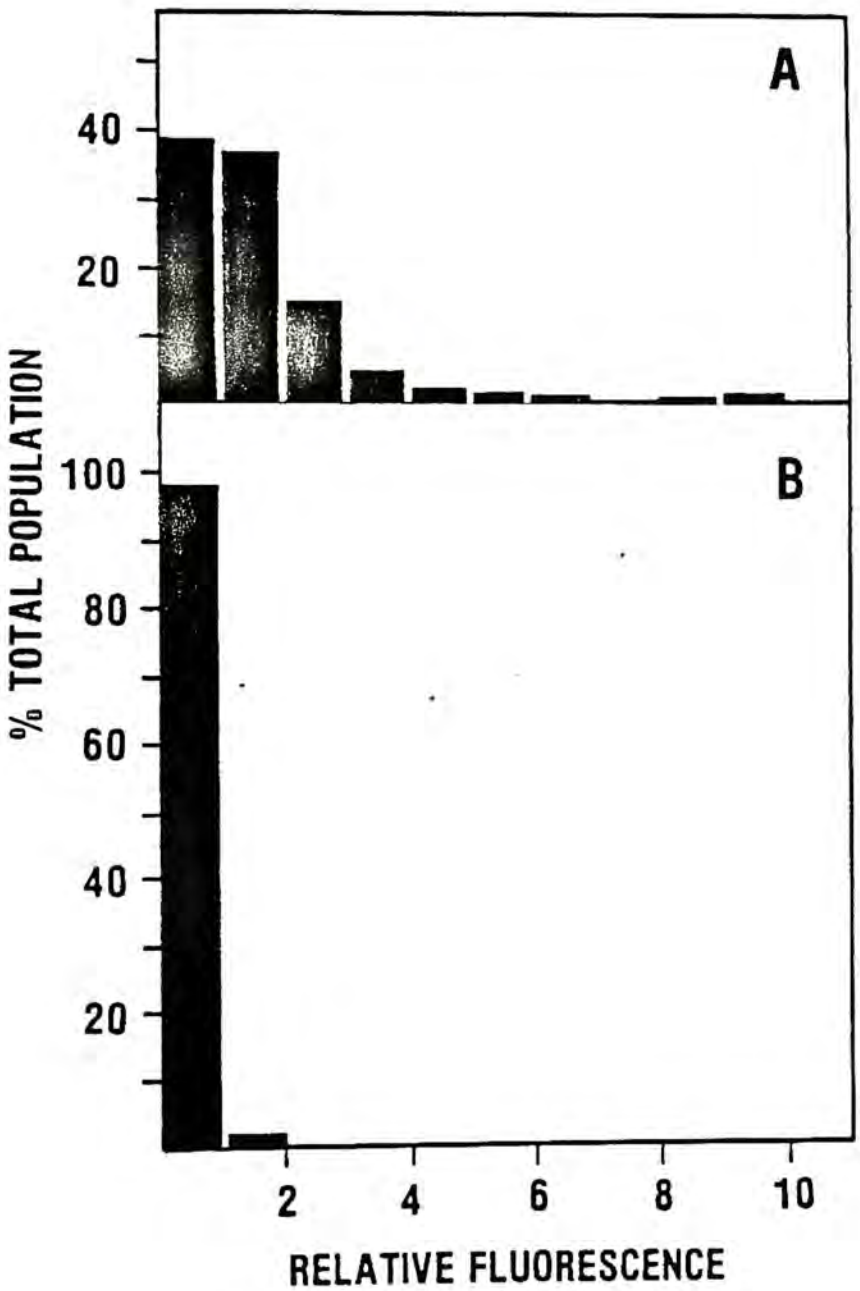
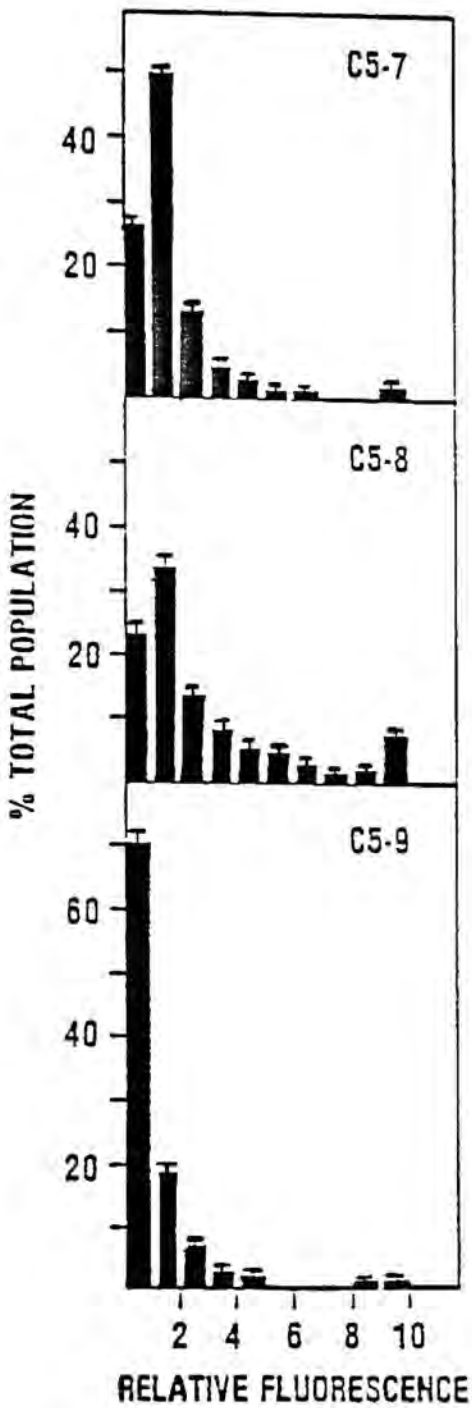

A
B
40
20
100
80
60
40
20
% TOTAL POPULATION
2
4
6
8
10
RELATIVE FLUORESCENCE

Figure 32

Fluorescence profiles of LUV's with C5-7 complexes, C5-8 complexes, and C5-9 complexes. The PMT setting for this set of experiments was increased to 51% for greater sensitivity. Each division on the x-axis represents 25 fluorescence units.


C5-7
C5-8
C5-9
40
20
40
20
60
40
20
% TOTAL POPULATION
2 4 6 8 10
RELATIVE FLUORESCENCE

higher proportion of more fluorescent vesicles relative to the C5-7 control. This increase in the number of more highly fluorescent vesicles occurred without a concomitant decrease in the number of vesicles per field. A decrease in the number of vesicles per field would be expected for all-or-none release or if C5-8 induced fusion of LUV's. Indeed, two fold changes in particle density are readily detectable. The result obtained was consistent with the increase in the Fo/Ft ratio of C5-8-treated LUVs, which in turn is indicative of graded release (Table XVII). In contrast, Fo/Ft of the C5-9-treated vesicles was the same as the control (C5-7) vesicles, indicating all-or-none release. Thus, analysis of complement-treated LUV's by the ACAS 470 system confirmed the graded and all-or-none modes of dye release from C5-8 and C5-9-treated vesicles, respectively, as determined by fluorescence studies.

Comparison of the $C5\text{-}9_1$ Complex to C5-8 and C5-9 Complexes on LUV's

Since C5-8 LUV's released dye in a graded fashion while C5-9 (under conditions where 6CF release was under 50%) released dye in an all-or-none manner, it was of interest to determine whether the mode of dye release depended on the number of C9 molecules bound per C5-8 complex. Studies by Bhakdi (50) had shown

Table XVII. Internal Fluorescence after 6CF Release from LUV's Induced by C5-8 and C5-9

Sample	% CF Release[a]	Predicted Fo/Ft for all-or-none release	Predicted Fo/Ft for Graded Release[b]	Measured Fo/Ft
C5-7	0 (control)	-	-	0.06 ± .01
C5-8	22	0.06	0.095	0.10 ± .01
C5-9	32	0.06	0.11	0.05 ± .01

a. Determined by following CF release to endpoint on a spectrofluorometer. 100% release is determined in the presence of Triton X-100.

b. Predicted on the basis of a quenching curve determined by the method of Blumenthal et al. (65).

that the number of C9 molecules per C5-9 complex was dependent on the temperature at which C9 was added to the C5-8 complex. In particular, Bhakdi showed that at 4 °C, one C9 was bound per C5-8 complex. To further understand the nature of the "C5-9_1" complex as defined by Bhakdi, C5-9 complexes were assembled at 4 °C and the resulting complexes were compared to C5-8 and C5-9 complexes assembled at 37 °C with respect to the mode of dye release and pore size. Table XVIII shows that, like the C5-8 complex, vesicles bearing "C5-9_1" complexes released dye in a graded manner. In molecular sieving experiments performed with entrapped tritiated sucrose or inulin as well as 6CF, "C5-9_1"-treated vesicles released an equivalent fraction of sucrose and 6CF but very little inulin (Table XIX). Futhermore, "C5-9_1" LUV's released much less inulin than vesicles on which C5-9 was assembled at 37 °C. The inulin release data indicate that, for the most part, C5-9 complexes formed at 4 °C have diameters too small for the passage of inulin. However, the small amount of inulin released might be explained if some of the complexes aggregated to form lesions of larger pore size. Although it was not possible to obtain an exact stoichiometry of C9 to C5-8 complex, the relative amount of C9 bound per C5-8 in the complexes assembled at 4 °C was approximately 70% of C9 bound per C5-8 in

Table XVIII. Internal Fluorescence After 6CF Release From LUV's Induced by C5-8, C5-9 and $C5\text{-}9_1$

Sample[a]	% CF Release[b]	Predicted Fo/Ft for all or none[c]	Predicted Fo/Ft for Graded Release[d]	Measured Fo/Ft[e]
C5-7	0 (control)	-	-	0.08
C5-8	38	0.08	0.11	0.11
C5-9	55	0.08	0.15	0.08
$C5\text{-}9_1$	52	0.08	0.14	0.15

a. Complexes assembled on LUV's loaded with 100 mM 6CF as described in Materials and Methods.

b. Determined by following 6CF release to endpoint on a spectrofluorometer. 100% release of 6CF was determined by addition of 2% Triton X-100.

c. Assumes some vesicles released all their dye; others released none.

d. Predicted on the basis of a quenching curve determined by the method of Blumenthal et al. (65).

e. Determined following the removal of released 6CF with a Sepharose-4B column.

Table XIX. Release of Encapsulated Sugars from LUV's Treated with C5-8, C5-9 and $C5\text{-}9_1$

	Sucrose-Loaded Vesicles		Inulin-Loaded Vesicles	
Sample[a]	% Sucrose Release[b]	% 6CF Release[c]	% Inulin Release[b]	% 6CF Release[c]
C5-7	0	0	0	0
C5-8	31.6 ± 0.1	30 ± 0.2	0	38 ± 0.65
C5-9	39 ± 0.15	40 ± 0.9	26 ± 0.2	52 ± 0.95
$C5\text{-}9_1$	42 ± 0.11	44 ± 0.1	9 ± 0.2	57 ± 0.55

a. Complement complexes were assembled on LUV's loaded with 100 mM 6CF and radiolabeled sugar as described in Materials and Methods. Samples are the average of duplicates.

b. Determined by comparing the residual ^{3}H-sucrose or ^{3}H-inulin associated with vesicle fractions to those of the C5-7 control following the removal of released ^{3}H-sugars with a Sepharose-4B column.

c. Determined by following 6CF release to endpoint on a spectrofluorometer. 100% release of 6CF was determined by addition of 2% Triton X-100 to vesicles.

the C5-9 complex assembled at 37 °C (Table XX). Thus, in contrast to results with RBC's, the number of C9 molecules bound per C5-8 complex on vesicles does not differ dramatically with assembly of C5-9 at 37 or 4 °C. Nevertheless, the higher temperature required to stabilize and enlarge the lesion formed by the C5-8 complex suggests a structural difference between the C5-9 complexes formed at 37 and 4 °C.

Temperature-Dependence of 6CF Release by C5-8 and C5-9 Complexes

The influence of temperature on C5-8 and C5-9 complex activity was determined by adding C8, or C8 and C9, to C5-7 LUV's and measuring rate and percent dye release at different temperatures ranging from 35 to 4 °C. Figures 33-38 show that both the rate and percent of 6CF release from C5-8- and C5-9-treated vesicles were temperature-dependent. The functional activities, however, did not show a strong dependence on lipid composition. Activation energies (Ea) for the C5-8 and C5-9-mediated dye release from vesicles of different lipid composition were calculated from Arrhenius plots (Figures 39-41). Surprisingly, despite the differences in the rates of C5-8- and C5-9-mediated dye release at every temperature studied, the activation energies associated with 6CF release were

TABLE XX. Comparison of the Amount of C9 Bound per C5-8 for C5-9 Complexes Assembled at 4 °C and 37 °C[a].

Exp[b]	Ratio of C9 Bound at 4 °C to C9 Bound at 37 °C[c]
1	0.71 ± 0.04
2	0.69 ± 0.03

a) C5-9 complexes were assembled on LUV's loaded with 100 mM 6 CF at 4 °C and 37 °C as described in Materials and Methods. Vesicle samples were then pelleted at 100,000 x g for 30 minutes, run on 7.5% polyacrylamide slab gels under non-reducing conditions, and transferred to nitrocellulose paper by electroblot electrophoresis. The blots were immunostained with anti-C9 and anti-C6 antibodies and the relative amount of proteins bound were determined densitometrically as described in Materials and Methods. The amount of C9 bound per C5-8 was determined by the ratio of the relative amount of C9 bound to the relative amount of bound C6.

b) One and 2 represent the results of two experiments.

C) The ratios shown represent the average of duplicate samples.

Figure 33

Dependence of the Functional Activity of C5-8 Complexes on Temperature: C5-7 complexes were assembled on DML vesicles containing 100 mM 6CF as described in Materials and Methods. C8-mediated dye release from C5-7 LUV's was monitored at different temperatures. Sample points are the average of duplicates.

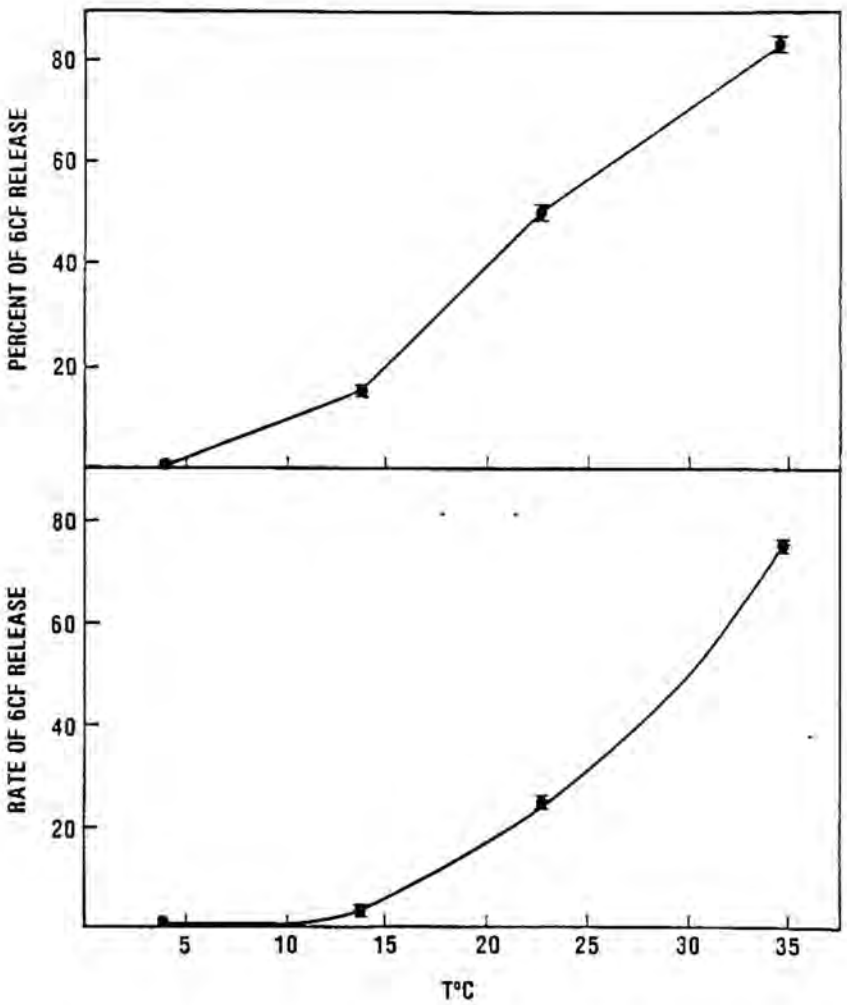

PERCENT OF 6CF RELEASE
80
60
40
20
RATE OF 6CF RELEASE
80
60
40
20
5
10
15
20
25
30
35
T°C

Figure 34

Dependence of the Functional Activity of C5-9 Complexes on Temperature: C5-7 complexes were assembled on DML vesicles containing 100 mM 6CF as described in Materials and Methods. C9-mediated dye release from C5-7 LUV's was monitored at different temperatures. Sample points are the average of duplicates.

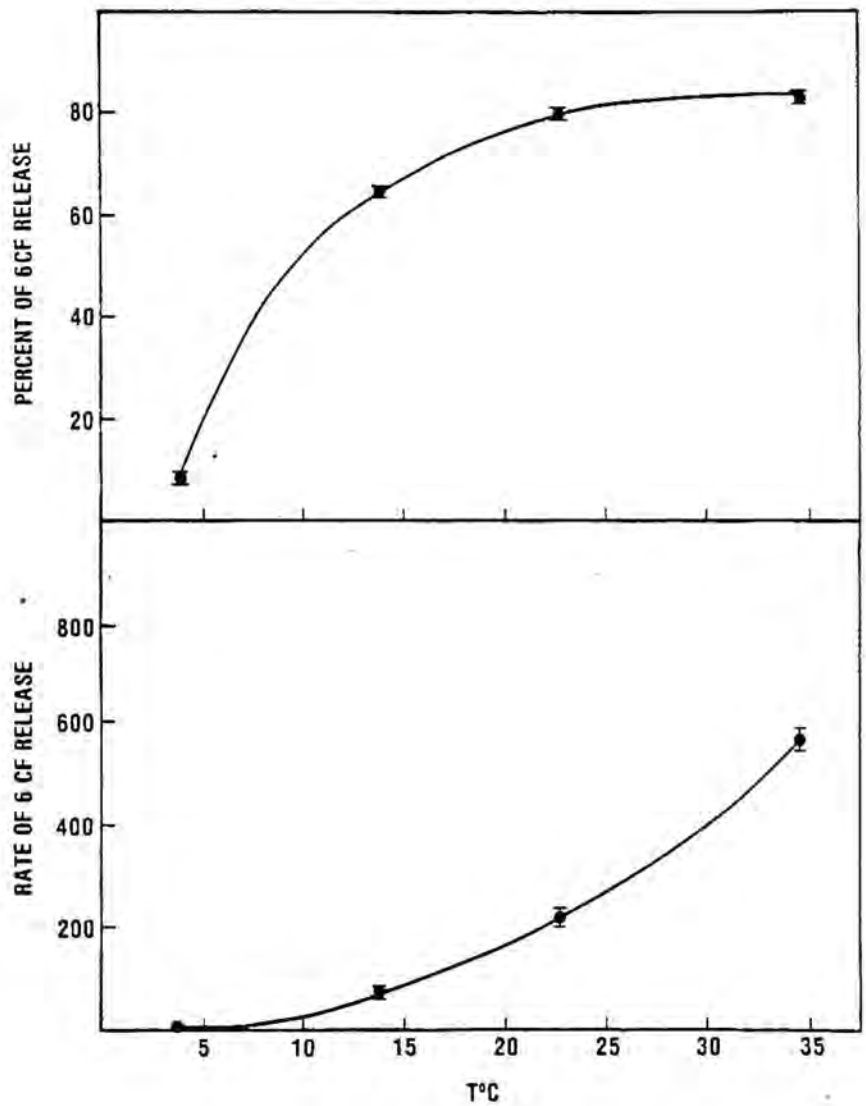

PERCENT OF 6CF RELEASE
80
60
40
20
RATE OF 6 CF RELEASE
800
600
400
200
5
10
15
20
25
30
35
T°C

Figure 35

Dependence of the Functional Activity of C5-8 Complexes on Temperature: C5-7 complexes were assembled on EYL vesicles containing 100 mM 6CF as described in Materials and Methods. C8-mediated dye release from C5-7 LUV's was monitored at different temperatures.

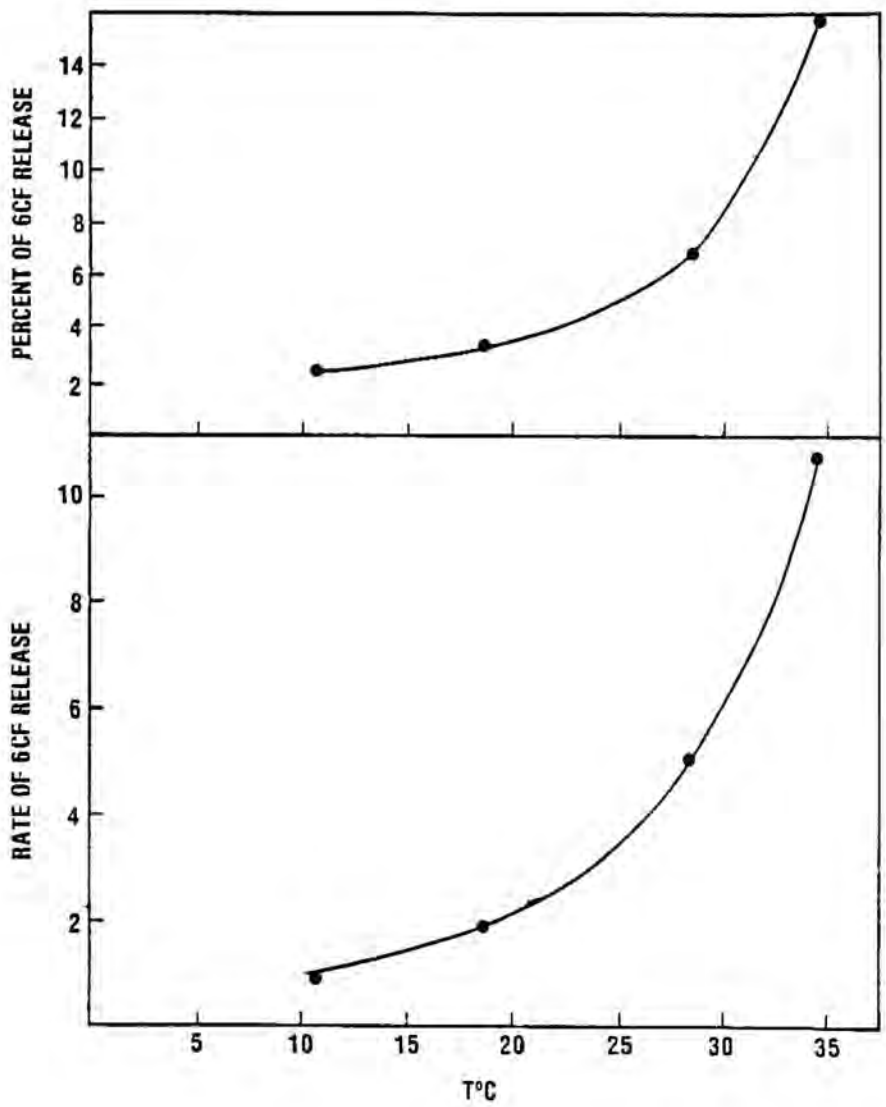

PERCENT OF 6CF RELEASE
14
12
10
8
6
4
2
RATE OF 6CF RELEASE
10
8
6
4
2
5
10
15
20
25
30
35
T°C

Figure 36

Dependence of the Functional Activity of C5-9 Complexes on Temperature: C5-7 complexes were assembled on EYL vesicles containing 100 mM 6CF as described in Materials and Methods. C9-mediated dye release from C5-7 LUV's was monitored at different temperatures.

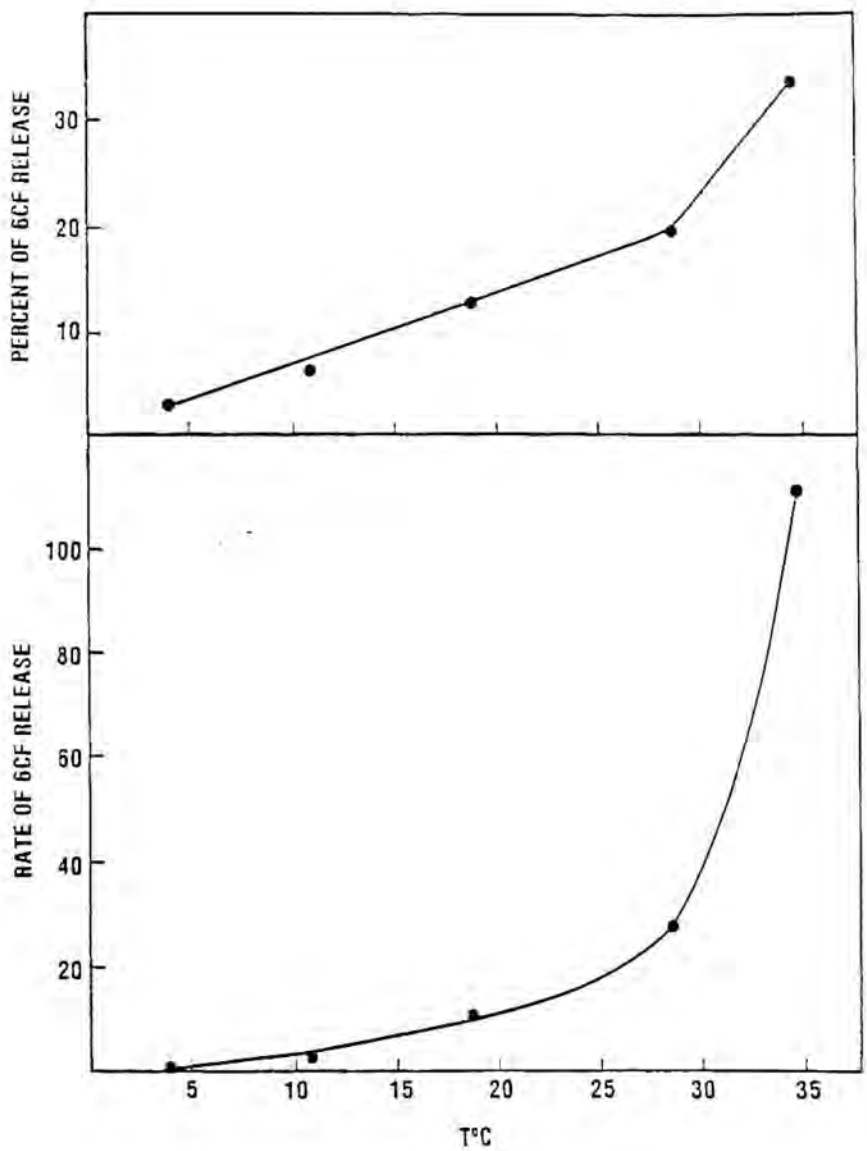

PERCENT OF 6CF RELEASE
30
20
10
RATE OF 6CF RELEASE
100
80
60
40
20
5
10
15
20
25
30
35
T°C

Figure 37

Dependence of the Functional Activity of C5-8 Complexes on Temperature: C5-7 complexes were assembled on DPL vesicles containing 100 mM 6CF as described in Materials and Methods. C8-mediated dye release from C5-7 LUV's was monitored at different temperatures. Sample points are the average of duplicates.

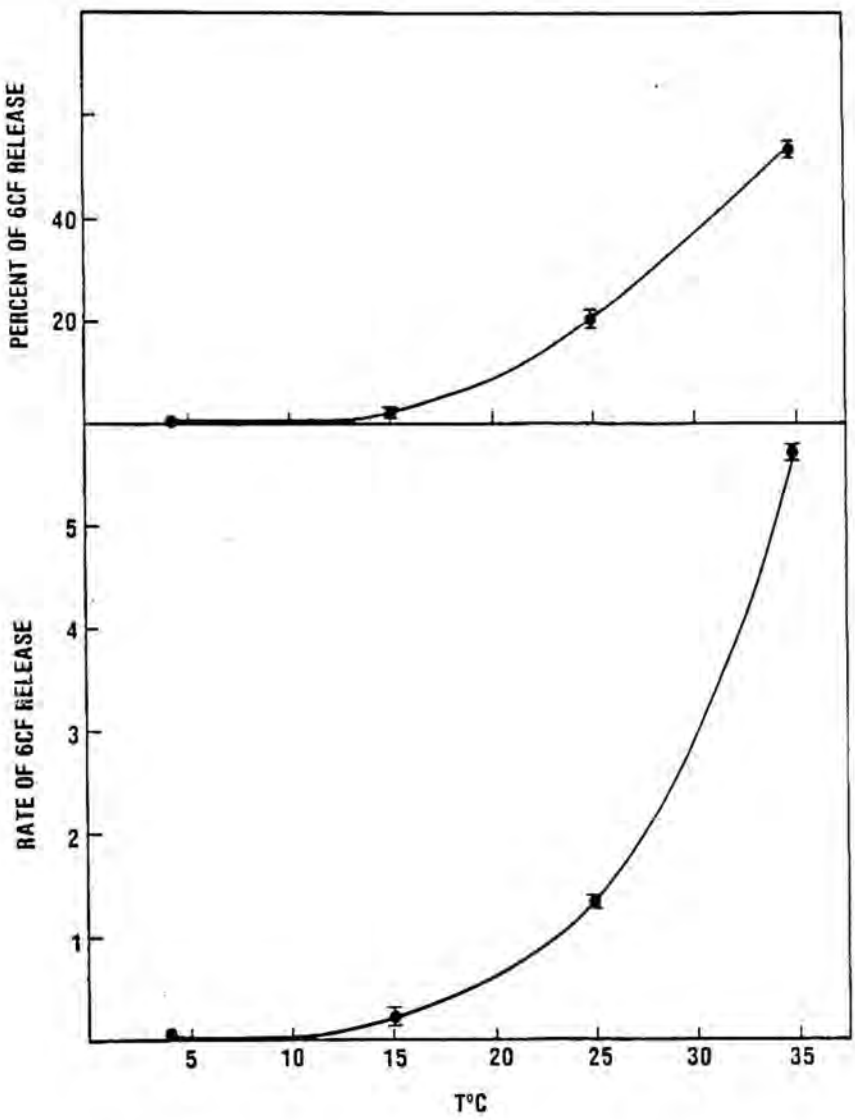

PERCENT OF 6CF RELEASE
40
20
RATE OF 6CF RELEASE
5
4
3
2
1
5
10
15
20
25
30
35
T°C

Figure 38

Dependence of the Functional Activity of C5-9 Complexes on Temperature: C5-7 complexes were assembled on DPL vesicles containing 100 mM 6CF as described in Materials and Methods. C9-mediated dye release from C5-7 LUV's was monitored at different temperatures. Sample points are the average of duplicates.

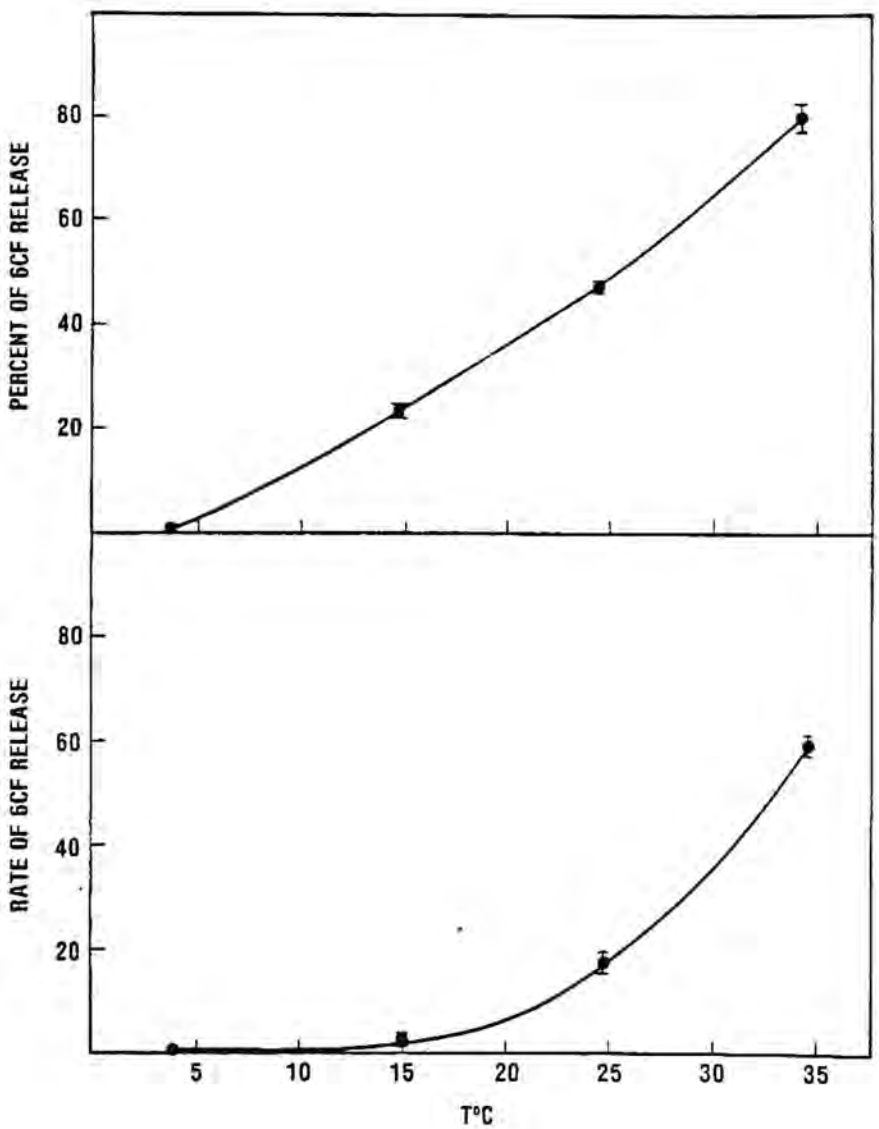

PERCENT OF 6CF RELEASE
80
60
40
20
RATE OF 6CF RELEASE
80
60
40
20
5
10
15
20
25
30
35
T°C

Figure 39

Arrhenuis Plot for C5-8 and C5-9-mediated 6CF Release from DML Vesicles: The rates of 6CF release (k) were derived as described in Materials and Methods. The X-axis indicates reciprocal of absolute temperature (K^{-1}). Data were compiled from two separate experiments each involving duplicate samples. Circles: C5-8-treated vesicles; Squares: C5-9-treated vesicles. Lines drawn indicate the best fit derived by linear regression of data.

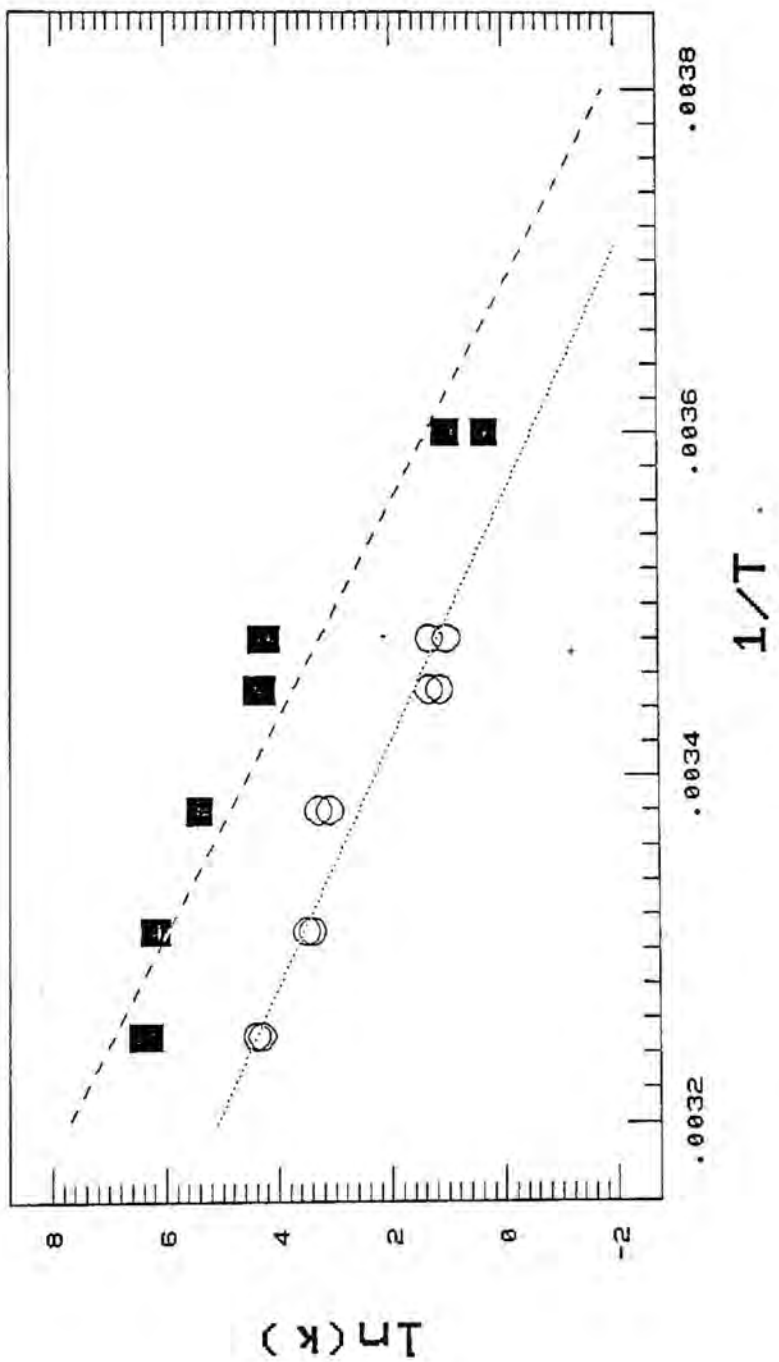

8
6
4
2
0
-2
.0032
.0034
.0036
.0038
1/T
ln(k)

Figure 40

Arrhenuis Plot for C5-8- and C5-9-mediated 6CF Release from EYL vesicles: The rates of 6CF release (k) were derived as described in Materials and Methods. The X-axis indicates reciprocal of absolute temperature (K^{-1}). Data were compiled from two separate experiments each involving duplicate samples. Solid triangles: C5-8-treated vesicles; open triangles: C5-9-treated vesicles. Lines drawn indicate the best fit derived by linear regression of data.

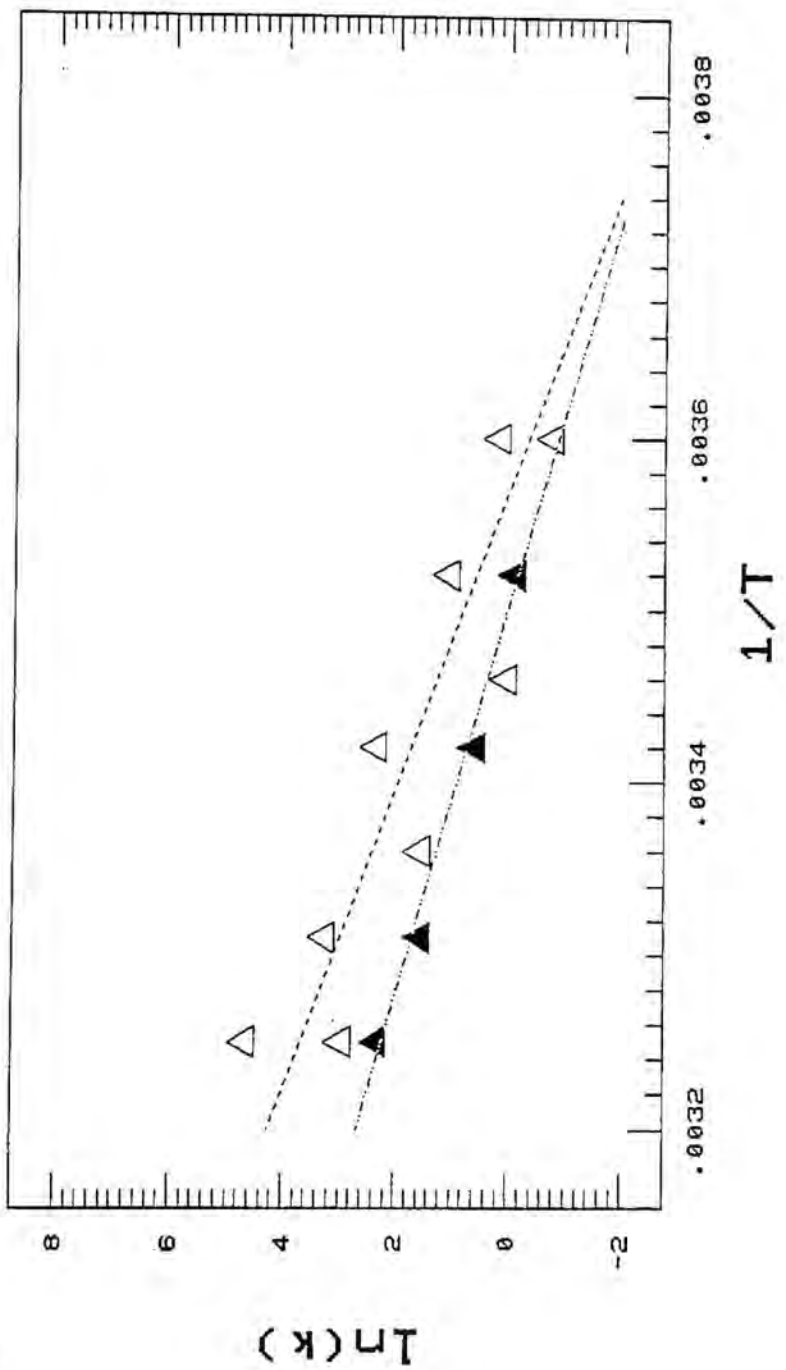
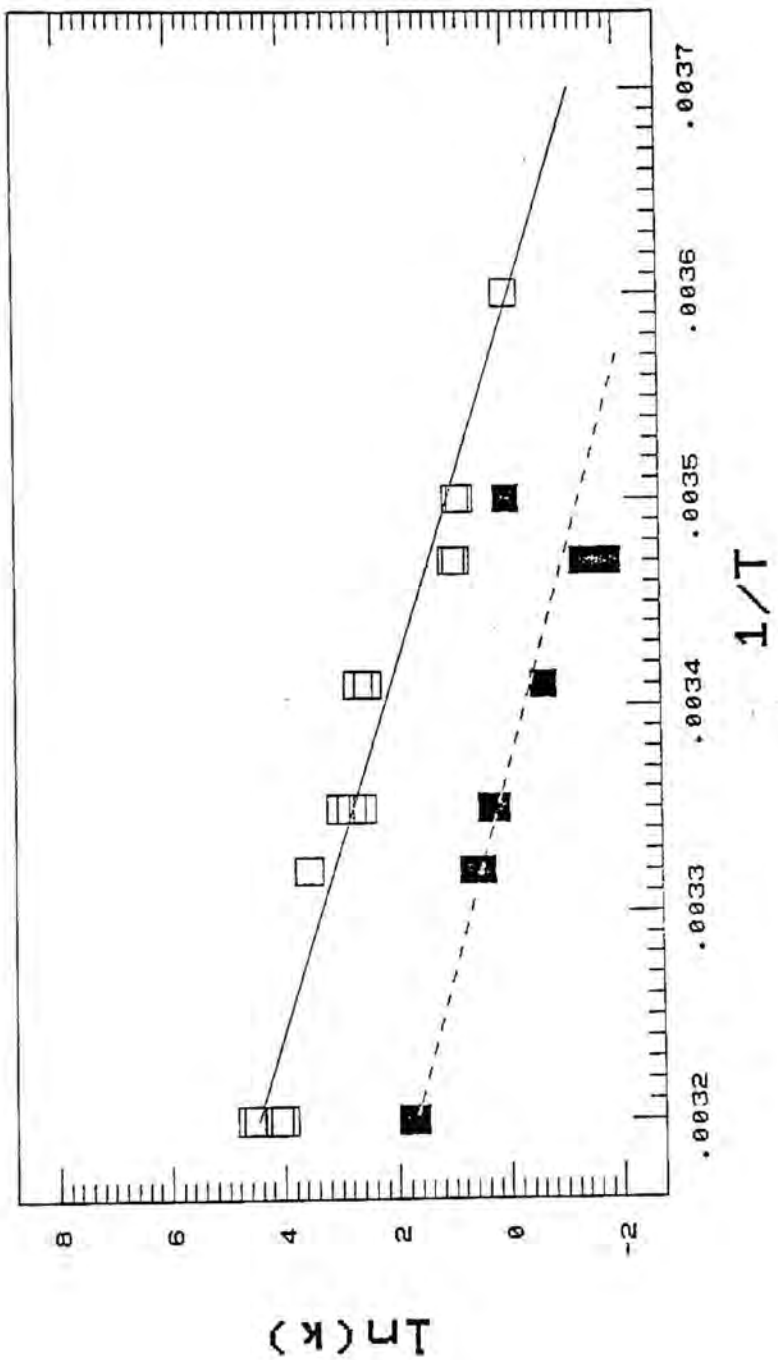

8
6
4
2
0
-2
ln(k)
.0032
.0034
.0036
.0038
1/T

Figure 41

Arrhenuis Plot for C5-8- and C5-9-mediated 6CF Release from DPL Vesicles: The rates of 6CF release (k) were derived as described in Materials and Methods. The X-axis indicates reciprocal of absolute temperature (K^{-1}). Data were compiled from two separate experiments each involving duplicate samples. Solid squares: C5-8-treated vesicles; open squares: C5-9-treated vesicles. Lines drawn indicate the best fit derived by linear regression of data.


8
6
4
2
0
-2
Ln(k)
.0032
.0033
.0034
.0035
.0036
.0037
1/T

similar for the C5-8 and C5-9 complexes (Table XXI). Furthermore, the activation energies for dye release by C5-8 and C5-9 complexes was not heavily influenced by different lipid compositions.

Comparison of the Insertion of C8 and C9 into LUV Membranes to Functional Complement Complex Activity

It was of interest to compare functional activity to a molecular process associated with the formation of C5-8 and C5-9 functional lesions. The insertion of C8 and C9 into vesicle membranes was studied since it had been previously shown that insertion could be correlated with 6CF release from vesicles and with lysis of RBC's (41, 43 and 44). In studying the process of channel formation by the C5-8 and C5-9 complexes, we were particularly interested in whether 6CF release occurred simultaneously insertion, or whether a post-insertional process was involved. To address this question, both the kinetics and temperature-dependence of insertion was investigated and compared with the kinetics and temperature-dependence of 6CF release determined in the aforementioned studies.

For the kinetic studies, C5-7 was assembled on LUV's containing the membrane-restricted probe, 12-APS-GlcN. Photoactivation of the probe took place at

TABLE XXI. Activation Energies for C5-8- and C5-9-Mediated Dye Release from Vesicles of Different Phospholipid Composition

LUV's (Phospholipid Composition)[a]	Activation Energy (Kcal/mol)[a] C5-8	C5-9
DML (standard vesicles)	27.6	31.4
EYL	17.5	22.8
DPL	19.8	22.5

a. LUV's composed of different phospholipids in place of DML were prepared by reverse phase evaporation as described in Methods. The same molar ratio's of phospholipid to cholesterol and DCP were used to prepare EYL and DPL vesicles as were used to prepare DML LUV's.

b. Activation energies were calculated from Arrhenius plots of data obtained from experiments performed as described in Figures 37-39. The form of Arrhenius equation used to determine activation energy was:

$$\ln k = \frac{Ea}{2.303\ R\ T} - \frac{1}{} + \ln A;$$

$R = 1.987$ Cal deg^{-1} $mole^{-1}$;
Ea = activation energy;
T = temperature (° Kelvin);
A = pre-exponential factor.

various times after the addition of C8 to C5-7 LUV's at 37 °C. The degree of C8 insertion as a function of time after C8 addition was determined after electrophoresis of the solubilized membrane pellets and autoradiography. As shown in Table XXII, insertion was completed within 30 secs after the addition of C8 to C5-7 LUV's at 37 °C. In contrast, 6CF release from C5-8-treated vesicles occurs over a period of at least 120 secs after C8 addition. Table XXIII shows the dependence of C8 and C9 insertion on temperature. The results indicate that the insertion of C8 was not significantly temperature-dependent, despite the fact that the rate and percent of 6CF release from C5-8-treated vesicles was strongly temperature-dependent. In particular, the insertion of C8 at 4 °C was ≈ 96% of that at 37 °C even though 6CF release is completely inhibited at 4 °C. The insertion of C9 at 4 °C was 68% of that at 37 °C. However, even though C9 insertion exhibited some temperature dependency, this does not explain the complete inhibition of 6CF release at 4 °C. These results imply that insertion of C8 and C9 cannot account completely for process(es) involved in channel formation; the release of dye from complement-treated vesicles most likely involves an additional post-insertional process(es).

Table XXII. Insertion of C8 into LUV Membranes as a Function of Time Following the Assembly of C5-8 Complexes on Vesicles[a]

Time (sec.)[b]	Insertion of C8[c]
0	1.19
30	1.39
60	1.26
120	1.08
300	1.08
600	1.15

a. The photoreactive probe, 12 APS-GlcN, was incubated with LUV's for 15 minutes at 37 °C before C5-8 complexes were assembled on vesicles. C5-8 complexes were assembled on LUV's as described in Materials and Methods C8 was incubated with C5-7 vesicles for different times before irradiation of samples. LUV samples were then pelleted at 180,000 x g for 30 minutes, run on 7.5% polyacrylamide slab gels under non-reducing conditions and transferred to nitrocellulose paper by electroblot electrophoresis. After staining with Amido Black, protein bands were cut out of the nitrocellulose paper and counted in a liquid scintillation counter to obtain the amount of radioactivity associated with each protein.

b. Time elapsed following the addition of C8 to C5-7 LUV's before irradiation of the vesicles. The "0" time point included a 2-4 second mixing time. Irradiation time was 10 sec.

c. C8 insertion was expressed as the ratio of the radioactive counts in the C8 band on the blot to the radioactive counts in the C6 bands. The data represent average values from duplicate samples whose S.E. were within 10%.

Table XXIII. C8 and C9 Insertion as a Function of Temperature

Temperature °C[a]	Insertion of C8[b]	Insertion of C9[b]
4	1.09	1.87
23	1.21	2.47
37	1.14	2.76

a. The photoreactive probe, 12 APS-GlcN was incubated with LUV's for 15 minutes at 37 °C before C5-8 and C5-9 complexes were assembled on vesicles. C5-8 and C5-9 complexes were assembled on LUV's as described in Materials and Methods. C8, or C8 and C9, were incubated with C5-7 vesicles at different temperatures for 15 minutes before irradiation of samples. Samples were then pelleted at 180,000 x g for 30 minutes, run on 7.5% polyacrylamide slab gels under non-reducing conditions, and transferred to nitrocellulose paper by electroblot electrophoresis. After staining with Amido Black, protein bands were cut out of the nitrocellulose paper and counted in a liquid scintillation counter to obtain the amount of radioactivity associated with each protein.

b. C8 and C9 insertion was expressed as the ratio of the radioactive counts in C8 or C9 to the radioactive counts in C6.

DISCUSSION

In a number of ways, the behavior of the terminal complement complexes on model membranes was analogous to the behavior of these complexes on red blood cells, suggesting the appropriateness of this model system for studying the interaction of the terminal complexes with membranes. The entire terminal complex consisting of either C5-8 or C5-9 must be assembled on RBC membranes in order for lysis to occur; the assembly of C5-7 alone on red blood cells does not cause lysis (76). Likewise, the full assembly of C5-8 or C5-9 on vesicles is required for 6CF release. Another common feature is the slow rate of hemolysis (14-16) or dye release induced by C5-8 compared to that induced by the C5-9 complex. Furthermore, the pore sizes determined by molecular sieving experiments for the C5-8 and C5-9 complexes on LUV's compare very well to their respective pore sizes on RBC's (32). C5-8 complexes on both membranes allowed sucrose but not inulin to pass suggesting a pore size of at least 8 Å in diameter but not greater than 30 Å. C5-9 complexes, on the other hand, permit the passage of both sucrose and inulin indicating that the C5-9 lesion is equal to or greater than 30 Å in diameter. Finally, lysis of RBC's and dye release from LUV's treated with

complement complexes are temperature-sensitive. Although both C8 and C9 bind to C5-7 on both types of membranes at 4 °C, neither lysis nor dye release occurs at this temperature. Only at higher temperatures are lysis (77) and dye release observed.

These studies using model membranes not only demonstrated similarities between the behavior of complement on RBC and LUV's, but provided new information concerning the nature of both the C5-8 and C5-9 membrane lesions with respect to the lifetime or stability of the channels and the involvement of a post insertional event involved in the formation of the channels.

The present studies on the mode of marker release from vesicles treated with C5-8 and C5-9 indicate that these "channels" may be mechanistically different, since the C5-8 complex induces only a transient permeability change in the membrane whereas the C5-9 complex generates an apparently stable and large-sized pore. The combination of smaller pore size and transiency of C5-8 complexes may explain both the slower kinetics and lower extent of C5-8-induced marker release from vesicles as well as the slower kinetics and lower extent of lysis observed with RBC's treated with C9-deficient serum (17).

The cause of the instability of the C5-8 lesion is not known but several explanations are plausible. If the C5-8 complex forms a structural channel in the membrane, a conformational change in the protein complex may either close or reduce the size of the pore. Indeed, Michaels et al. (25) have shown that the C5-8 channel, but not the C5-9 channel, is voltage-sensitive in black lipid membranes. Specifically, trans-negative voltages increased the conductance through C5-8 channels, while trans-positive voltages decreased the conductance. It is possible, therefore, that a trans-positive membrane potential, perhaps induced by the efflux of negatively-charged 6CF from the vesicle, could induce a conformational change in the C5-8 complex which would effectively close the channel before all the dye could leak out. However, this explanation would not apply to the graded release of uncharged markers such as sucrose. The fact that the percentage of sucrose release by C5-8 always paralleled that of 6CF release in these experiments may be explained by the co-release of 6CF from the same vesicles.

Alternatively, it is possible that the C5-8 complex does not, in fact, form a structural channel in the membrane but rather induces a perturbation in the lipid bilayer. The trapped marker may leak out due to

a transient destabilization of the membrane during C5-8 assembly. Such a transient destabilization leading to phospholipid flip-flop has been noted previously during the incorporation of cytochrome b_5 into model membranes (78) and is suggested by the results with gramicidin. Furthermore, Dankert et al. (61) reported that C9 alone inserts readily into small but not large unilamellar vesicles and suggested that a loosely packed lipid bilayer was necessary for C9 insertion. The authors proposed then that the primary function of the C5-8 complex was to disorder the membrane and facilitate C9 insertion into the bilayer. While this hypothesis has some appealing aspects, the data presented in this study demonstrating that C9 can enhance lysis by C5-8 well after C5-8-induced marker release has ceased would argue against it, assuming the membrane was restabilized after the endpoint of C5-8-mediated dye release had been reached.

Finally, the state of aggregation of the C5-8 complexes in the membrane also needs to be considered as having a possible bearing on the functional activity of the C5-8 complex. Cheng et al. (59) have shown that the functional activity of C5-8 complexes on RBC's could be correlated with the state of aggregation of the C5-8 complexes on the cells. This interesting observation suggests that changes in the state of

aggregation of the complexes in the membrane could also have the opposite effect of abrogating C5-8 channel activity.

At present, it is not possible to definitively confirm or eliminate any of these possible explanations for an unstable C5-8 "channel." Nevertheless, the information presented in this study regarding C5-8 channel instability may help to explain why the C5-8 complex is relatively inefficient in lysing cells.

The conversion of an unstable complement channel to a stable one apparently requires the assembly of the C5-9 complex at higher temperatures. Bhakdi et al. (50) have shown that at 4 °C, only one C9 molecule binds per C5-8 complex whereas approximately 6 C9 molecules per C5-8 complex are bound at 37 °C. While we have not been able to observe this difference in C9 stichiometry on vesicles, the C5-9 complex generated at 4 °C behaves more like the C5-8 complex than the C5-9 complex assembled at 37 °C, both in terms of "pore size" as well as mode of dye release (i.e., graded). This surprising result indicates that the usual binding of C9 molecules to C5-8 at physiological temperatures serves a very practical purpose, that of stabilizing and enlarging the membrane lesion initiated by the C5-8 complex. At present, the structural

differences between the C5-9 complexes generated at 37 and 4 °C are not known.

While the functional activity of C5-8 and C5-9 complexes has been associated with the insertion of these proteins into the bilayer of both model and natural membranes (41, 43, 44), the rate-limiting step(s) for the formation of the respective channels has not been determined. In this study, the relationship between complement protein insertion into membranes and channel formation (i.e., 6CF release) was investigated. The results presented here provide evidence for a post-insertional step(s) in the process leading to channel formation by both C5-8 and C5-9 complexes. This evidence, in part, involves the observation that C8 insertion was completed well before the endpoint of dye release, indicating that insertion of the terminal proteins, and in particular C8, is a necessary but insufficient condition for channel formation. This interpretation is strengthened by the finding that, at 4 °C, no 6CF was released from vesicles treated with C5-8 or C5-9 even though at least 96 and 68% of C8 and C9, respectively, was inserted into these membranes at this temperature. Although the argument could be raised that at the lower temperatures, improper insertion of C8 and C9 might occur leading to dysfunctional C5-8 and C5-9 complexes,

the kinetics of C8 insertion relative to the kinetics of dye release and the temperature-insensitivity of C8 and, to a lesser extent, C9 insertion collectively suggest that a post-insertional event is involved in the release of 6CF from C5-8- and C5-9-treated vesicles.

The nature of this post-insertional step has not been defined but the activation energies (Ea) determined for 6CF release by C5-8 and C5-9 complexes raise interesting possibilities. Sims and Wiedmer have previously reported the activation energies for C5-8-induced C9 polymerization on several types of vesicles as well as on RBC's (79). Remarkably, the activation energies for polymerization on vesicles correspond fairly well to the activation energies determined in this study for 6CF release by both C5-8 and C5-9 channels. Moreover, in both cases, the activation energies show only marginal dependence on lipid composition. It is indeed surprising that the activation energies for C5-8 and C5-9 channel formation are so similar, given the significant difference in the rate of dye release induced by these two complexes. It is intriguing to speculate that the rate-limiting step in channel formation may be the same for both complexes even though the final complexes exhibit quite different properties. In addition, the fact that the activation

energies for C5-8-mediated C9 polymerization on vesicles are comparable to those determined for dye release raise the possibility that this process is also linked to the same or similar rate-limiting step. This rate-limiting step may be either a post-insertional conformational change in the C5-8 complex or the association of inserted C5-8 complexes in the plane of the membrane.

The following then are possible structures for the C5-8 complex which are consistent with the results. C5-8 could form small, discrete structural channels on vesicles which exist in open and closed conformational states. The transient nature of the C5-8 channel might reflect conversion of all complexes to the closed conformation resulting in the inability to release total vesicle contents. The binding of C9 to the C5-8 complex may prevent the conformational change to the closed state. Alternatively, the release of dye from C5-8-treated vesicles might depend on the aggregation of C5-8 complexes. Low temperatures may inhibit channel formation or dye release by impeding the aggregation process. In this case, a change in the aggregational state may be responsible for "closing" the C5-8 channel, with the binding of C9 stabilizing the "open" state. Finally, it is also possible that C5-8 creates a perturbation in the membrane bilayer

instead of a bona fide channel. Dye release would result from a disturbance in the ordering of the lipid bilayer due to the insertion of the C5-8 complex into the membrane. The bilayer perturbation could be transient in nature but stabilized in the presence of C9.

In summary, the results presented indicate that the C5-8 complex forms a transient channel or perturbation in the membrane which is stabilized and enlarged by the binding of C9 molecules at 37 °C. Furthermore, evidence is provided which suggests that a post-insertional event is involved in the formation of functionally active C5-8 and C5-9 complexes.

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